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OBSERVATIONS ON CERTAIN MARINE ORGANISMS OF (*a*) VARIATIONS IN REACTION TO LIGHT, AND (*b*) A DIURNAL PERIODICITY OF PHOSPHORESCENCE

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The observations recorded in this paper were chiefly conducted upon organisms taken by means of a fine silk tow-net in Port Erin Bay during the spring and summer of 1908.[1] In addition certain observations are added upon the reaction to light of young larvae of the plaice (*Pleuronectes platessa*) taken from the Hatchery of the Station.

The experiments on the action of light were made in April, and the attempt in September to investigate the action of light upon the phosphorescent organisms then present in the Bay, based on the supposition that organisms which themselves emitted light might possibly show interesting variations in reaction to incident light from without, led to the accidental discovery of the diurnal periodicity in the phosphorescence of these organisms, which furnishes the subject of the second section of this paper.

The two sets of experiments on the variations in relation to light, and upon the diurnal periodicity in phosphorescence, are really distinct, and will be described in two separate sections.

A.—Variations in the Reactions of Organisms (Chiefly Nauplii of Balanus) to Daylight and Artificial Light

Since the very existence of all living organisms, either directly or indirectly is dependent upon the energy of light, and the transformation of this into other types of energy, it is not surprising that reactions to light are amongst the most fundamental and most widely spread throughout the whole world of organized living creatures. Such reactions must have been developed in the very beginning of the dawn of life when the first living cells commenced to synthesize organic products from the inorganic materials of their environment by the use of the store of energy from the sunlight. Later on organisms arose which were only

1. All the tow-nettings used in Section B were taken in Port Erin Bay and were surface tow-nettings, some of those used in Section A were kindly taken for me outside the Bay by Prof. Herdman

dependent upon the light at second-hand, since they were able to consume the synthesized organic products formed by other organisms converting the light energy directly, and so were only indirectly dependent upon the light for their existence. Even for this type of organism, utilizing the light energy indirectly, reactions to light remained essential in the search for food and for other physiological functions, and also there would be an inheritance of relationships to light derived from the earlier ancestry with direct dependence upon light.

At a later stage structures or organs arose specially adapted for light reactions, and in those living creatures possessing such organs there probably came a deterioration of the sensitiveness to light of the remaining cells of the body. But in spite of all such decline in direct sensitiveness to light, there must have remained some trace of their old primeval relationships to light.

Experimental evidence of this persistence of relationship to light of all cells exists of two kinds; there is first the deleterious effects of complete withdrawal of light for prolonged periods, and the necessity of sunlight for healthy existence; and, secondly, there is the direct evidence of the effects of application of strong light to animal cells seen in the Finsen effects, and in other forms of radiant energy allied to light.

It is, however, in the more lowly organized types of both animal and vegetable organisms that the strongest and most direct reactions to light are observable—apart from the particular case of the reaction of chemical synthesis in the chlorophyll-containing cells of the green parts of the higher plants.

Examples of this reactivity are seen in the effects of sunlight upon nearly all types of bacteria; in the sudden outburst of vegetable life in the form of diatoms in the spring of each year as the length of the day increases and the more vertical light reaches and penetrates the water before there is much increase in the temperature of the sea—an outburst upon which the whole life of the sea is as thoroughly dependent as that of the terrestrial world is upon the similar outburst of activity in land plants; and in the most marked movements which occur towards or from the light according to varying circumstances of the minute organisms, either larval or adult, which chiefly constitute the plankton or floating life of the ocean.

It is hence clear that the observation of the reactions of living cells to light is of importance both to the student of biology, and to the student of medicine who makes practical applications of the discoveries of biology, using the term in its widest sense.

Recent discoveries have proven the value of light treatment as a practical adjunct of medicine, and the study of light effects upon the simpler organisms must sooner or later yield a key, both for the rational understanding of such effects, and their extension to further utility. In addition to these utilitarian advantages, the study is one of the most fascinating from its own intrinsic interest in the whole wide field of biology.

One of the most obvious lines of attack in investigating the reactions of living organisms to light is the study of the movements of the organisms, either as a whole in the case of freely moving organisms, or the change in relative position, or orientation, in fixed or sessile organisms.

It must, however, be clearly borne in mind that this movement is an index of other things, that the underlying problem is ultimately and essentially a chemical one, or, better expressed, one of chemical transformation of light energy.[1] The organisms move because of an action of light upon chemical constituents in the cells, that is to say, there is a change in the metabolism of the cell stimulated, giving rise to the movement of the organism. Also, according to the nature and condition of both cell and light-stimulus, which form the two inter-acting factors, the character and sense of the movement of the organism will vary. Thus, we shall see that with the same condition and previous treatment of the organism, the reaction varies and becomes positive or negative with varying intensities of the light-stimulus, and, secondly, with the same constant intensity of light-stimulus, the reaction varies when the previous history and condition of the reacting organism have been artificially varied. That is to say, the light induces chemical alterations in the cell, and the nature and amount of the chemical changes vary with the two factors, the condition of the cell at the time, and the intensity of the incident light.

It has been clearly pointed out by Loeb that the orientations or tropisms of sessile organisms, and the movements of free organisms towards or away from light, are essentially the same in character, the free organism being first orientated and then, by the action of its locomotor organs, carried in either direction according to the sense of the previous orientation.

This is a fundamental observation which to a certain extent unifies the problem, but there still remain the questions of why the light induces

1. This view has been also put forward by Loeb, *Dynamics of Living Matter*, 1906, pp. 112 *et seq.*

orientation, the conditions under which orientation varies with the condition of the organism and the strength of stimulation, and also the remarkable fact that in higher organisms at any rate there is developed what might be described as *resistance* to orientation, so that the organisms accumulate either at the proximal or distal point to the light and yet lie in all possible planes of orientation, and, further, that they move about within a certain zone in all possible directions.

It is in fact self-evident, and may be taken as axiomatic, that there must have been a certain degree of orientation, or steering, or the organisms would never have been able to move either to or from the light. But this, it is to be observed, is quite different from the organism being turned round when the movement first begins, being definitely held there by the influence of the light in a fixed plane, and then as a result moving towards or away from the light.

The experiments to be recorded later show clearly that there is no such fixed or rigid orientation keeping the organisms in a constant plane, but rather a continually directed control bringing the organism back more or less towards the same direction as it darts about under other varying influences and stimuli, and this on the whole gives steering to the course, so that the animal as a net result moves towards or away from the light.

Taking this movement then as a sign of chemical change in the cells of the organism, or certain of those cells, the effects were observed—of exposure of organisms to light of varying intensities, of change in reaction as a result of keeping in light of about constant intensity, of velocity of movement in light of varying intensity, of the effect of light of different colours, and of velocity of movement in such lights, of the effects of converging and diverging light, of the effects of light and shade on organisms in the same vessel, on the association of upward or downward movements in level with positive or negative phototaxis, and on movement in presence of more than one source of light.

A very considerable literature exists dealing with heliotropism and phototaxis, but no attempt need be made to quote from this, further than relates to the organisms used for the research, or in incidental relationship to the variations in reaction to light described in the present experiments.[1]

The experiments were made with a free-swimming larval stage of the Barnacle (Nauplii of Balanus), obtained in by far the largest quantity in

1. For a general survey and for literature, reference may be made to Verworn, *General Physiology*, translation by Lee, 1899; Holt & Lee, *American Journal of Physiology*, Vol. IV, p. 460, 1901; and Loeb, *Dynamics of Living Matter*, 1906.

the tow-nettings, mixed with a much smaller number of copepods, and larval spirochaetes

The manner in which the organisms congregate at the points of the dish nearest to and farthest from the light was used to pipette them off and separate them from other organisms indifferent to the light, and the positive and negative groups of organisms so obtained were examined separately. Many of the Nauplii were found in both the positive and negative groups, but no difference in average size or degree of development could be found in the two types to differentiate them, and later experiment showed that the same separated group might be artificially varied backward and forward between positive and negative according to their previous treatment by light.

The phototaxis of the Nauplius of Balanus has been examined by Loeb, and Loeb and Groom, and Loeb[1] states that they are positively heliotropic upon leaving the egg, but soon become negatively heliotropic. This I consider is entirely due to over-stimulation by the light, for on keeping for some time in darkness the negative organisms become strongly positive to the same intensity of light in which they were previously negative, and in which part of them left during the same interval have continued negative. The statement of Loeb and Groom that they remain positive in artificial light (gas flame) is confirmed by the results of my experiments, but holds up to a certain intensity of illumination only, for if the light of a small lamp was converged by means of a cylindrical museum jar in which the organisms were contained, the organisms in the strongly illuminated area gradually became negative and passed into the shaded parts or to the distal pole.

In later experiments made at Berkeley, U.S.A., Loeb found that Nauplii there behaved differently from those examined in his earlier experiments made at Naples, and showed more complicated reactions.

Working with the larvae of Polygordius, and with those of Limulus, Loeb noticed a phenomenon which was also conspicuous throughout the present series of experiments, namely, that the positively phototactic organisms gathered in a group towards the top of the vessel, while the negative organisms at the same time as they gathered away from the light congregated at the lower part of the vessel near the bottom.

This I have also invariably observed when a tow-netting is brought into the Station and placed in the diffuse light of a window in a glass jar. The positive organisms are in a compact group nearest to the window, and almost at the surface of the water; while the negative ones are at the most

1. *Loc. cit.*

distal point of the jar from the window and down near, or on, the bottom. The same arrangement holds even in a shallow dish, well illuminated throughout its depth, the positive organisms are up close to the surface, and the negative ones on the bottom of the dish. The arrangement continues when the organisms are lit in the dark room by a candle on the same level as the water still the positive ones are near the surface and the negative ones near the bottom.

I consider that the most probable explanation of this is the constant association, in the natural habitat of the organisms (the sea), of swimming upwards towards the light when positively phototactic, and downwards towards the darker regions of water when negatively phototactic.

In addition to the interest of this association on its own account, it seems to me to be valuable as a sign that the light not only affects the sensitive area on which it acts, but also indirectly affects the whole organism, the chemical changes set up at the sensitive area communicating changes to the whole organism, which stimulate it and cause it to rise or sink in the medium.

Loeb, working with Gammarus, found that traces of acid made the organisms more strongly positive, and traces of alkali tended to produce a negative heliotropic effect. I have not been able to obtain similar results with hydrochloric acid, or caustic soda, in Nauplius, although both reagents were pushed to the limits compatible with life, viz., $\frac{1}{500}$ normal.[1] The organisms in the dishes to which either acid or alkali was added seemed to behave exactly like the untreated control. I do not, however, consider this any contradiction of Loeb's results, since the organisms used were different. Moreover, Loeb's results are such as would be expected from the knowledge that alkalies, within the compatible range, excite the activity of living matter, while acids depress it. For, if we regard the light effect as producing an increased chemical activity, then the optimum value of reaction, at which the change would occur from positive to negative as the intensity of the illumination was increased, might be expected to be reached sooner in the case of an organism already made hyperactive by alkali than in the case of an organism where the activity was depressed by added acid.

Throughout the whole series of my experiments I have consistently found that the phototaxis is positive with very feeble illumination, and becomes negative as the strength of the light is increased. Further, continued illumination, either by diffuse daylight or by a very bright

1. The limit of acidity or alkalinity compatible with life seems to have *nearly* the above value for all unprotected minute organisms of either vegetable or animal origin.

artificial illumination, causes an increasing number of organisms to become negative, and keeping in darkness or in a feeble illumination causes this negativity to pass back to a positive phototaxis.

This again is compatible with the view that the effect of light upon the sensitive substances of the organism is always the same whether the effect is shown by a positive or negative phototaxis. The degree of the stimulus determines the reaction of the organism towards it, as shown by the direction of the orientation and consequent movement, but the chemical nature of the stimulus is the same. Below a certain optimum the organism reacts so that the sensitive surface is turned towards the light, that is to say so as to increase the amount of light energy reaching it, and so *increase* the reaction towards its optimum value for the organism in its condition at the given moment. Above the optimum value of stimulation, the organism conversely reacts so as to turn the sensitive surface into a region of diminished light intensity, and so also to *decrease* the velocity of the reaction towards its optimum for the organism.

This supports the view expressed by Holt and Lee,[1] that direction of light is only effective in a secondary manner in so far as it alters intensity of light falling upon different parts of the organism, and the orientation is hence primarily a question of intensity of light.

The very ingenious experiment of Loeb, showing that an organism which is positively phototactic to direct sunlight will pass from this onward into diffuse sunlight, that is, into a region of lower intensity of illumination, and will not reverse its direction when it finds itself in this region of lower illumination, is quite susceptible of explanation on this view, as well as the result of the experiments given below in this text, upon the movement of negatively phototactic organisms away from the source of illumination in converging light, and still onward past the focus of the light in now diverging light with decreasing intensity.

Loeb's experiment consisted in placing an organism (young caterpillars of *Porthesia chrysorrhaea*) in a test-tube the axis of which was horizontal and at right angles to the plane of a window near by, through the upper part of which direct sunlight fell on the more distal portion of the test-tube, while the portion of tube near the window was lit only by diffused daylight. Under such conditions these animals, which react positively, did not halt at the junction of diffuse light and direct sunlight and turn again backwards to the stronger light, but proceeded on in the feebler light toward the incident point right up to the end of the tube.

From this experiment, Loeb argues strongly against the anthropo-

1. *Loc. cit.*

morphic point of view which would assign any choice to the animal as to whether it sought, or turned from, the light because the light was pleasant to it or the reverse, and urges that the whole process is mechanical or automatic, the animal's head being turned by the stimulus irresistably towards the light, and the whole movement following inevitably upon this turning.

Without assuming any extravagantly anthropomorphic point of view, it may be maintained that the ingenious experiment scarcely supports the interpretation placed upon it, and that the whole matter depends upon the force of the stimulus outweighing the degree of development, of what represents the intelligence of the animal, or, if the expression is more suitable, the development of the nervous system, or, in more general terms still, the co-ordination of the organism.

When the animal's body or the sensitive area of it passes from the area of direct sunlight into the less illuminated area of diffuse daylight, in order to turn back into the brighter area of sunlight, the sensitive surface would require for a time to be turned away from even the diffuse light into a region of shadow from its own body, that is to say, it would require for the time to behave as a negatively phototactic animal, and reduce the intensity of illumination of the sensitive area. This supposes a degree of intelligence and of memory for the 'pleasanter' (or more near the optimum) stimulus which the organism does not possess, and hence it does not turn; but a more highly organized animal would turn, and once more seek the stimulus which suited the organism best.

It is such excess of stimulus over organization which makes the moth burn itself in the flame or the bird dash itself to pieces against the lighthouse lantern, and in my opinion this differs in degree of complexity only, but not in kind, from the strength of the irresistible impulse which forces the victim of any drug habit to keep on drugging himself, or leads the unfortunate human being with an incoordinated or improperly balanced nervous system into committing crimes against himself or others. The germs of resistance to stimuli, or rather of reacting so as to alter strength of stimuli, must be present in all living creatures, or life and continuance of the species would speedily become impossible; and it appears to me that denial of this would be nearly as great an error as the view which appears to be held by some opponents of the advance of physiological science, that all organisms and animals are about equally sentient to stimulation and to pain.

The experiments conducted with organisms under different coloured glasses, described below, in which the relationship of the two halves of the

dish to the direction of the incident light was identical, also show, from the selection of one-half of the dish by the organisms in preference to the other, that the organisms seek that region where the light activity possesses an optimum for them although there is nothing in the incident direction of light to lead them to swim under one particular glass as a result of orientation.

The same is seen in the experiments of Oltmann[1] and of Holt and Lee, in which a range of varying intensity of light was arranged by means of a prism placed along the long side of a long glass trough containing organisms. The incident light came in varying intensity perpendicularly through the prism, and the organisms were then found to place themselves in certain intermediate positions where the intensity of light suited their optimum, although they had to move to this position practically at right angles to the direction of incidence of the light.

Experiments were made in the present series of observations upon the velocity with which the organisms moved in light of varying intensity, and also under glasses of varying colour, and it was found that within the limits of the experiment, the velocity of movement was practically constant, thus showing that the chemical reactions set up by the light did not affect the locomotor organs.

Description of Experiments

Experiment I. A tow-netting was taken in Port Erin Bay, April 21st, 12—1 p.m. After stirring up in sea-water, it was divided into five portions of 300 c.c. each, which were placed in white soup plates and treated as follows:—

No. 1. Control, untreated.

No. 2.—Added 3 c.c. of $\frac{N}{10}$ HCl, making $\frac{N}{1000}$ solution.

No. 3.—Added 6 c.c. of $\frac{N}{10}$ HCl, making $\frac{N}{500}$ solution.

No. 4.—Added 3 c.c. of $\frac{N}{10}$ NaOH, making $\frac{N}{1000}$ solution.

No. 5.—Added 6 c.c. of $\frac{N}{10}$ NaOH, making $\frac{N}{500}$ solution.

The dishes were left in the diffuse daylight of a north window, and examined after two hours (3 p.m.), the arrangement of the organisms is

1. Quoted by Holt & Lee, *loc cit.*

found to be the same in all five plates, showing no change due to acid or alkali, and this persisted throughout the experiment.

In each of the dishes there are two prominent groups of organisms, a larger group at the part nearest the window and close to the surface of the water, a smaller, but well-marked group at the diametrical pole farthest from the window and at the bottom of the plate.

On shading, for a few minutes, half of one dish with a cardboard, the line of shade of edge of cardboard being at right angles to the plane of the window, in the illuminated half of the plate there is a thick group at the nearest point to the window; in the darkened semicircle, immediately on lifting the card, a smaller group is seen at the point distal to the light, and also there is a diffusely scattered but increased number over all this previously dark half, much greater than in corresponding areas of the illuminated half.

Examined again at night (8—9 p.m.) by lamp-light when nearly all the organisms in the plates come to the point nearest the light. Shading as before with shadow parallel to direction of incidence, gives a compact group in the illuminated half near the light, but a great many are in the darkened half which possesses a diffuse group at farthest point from light.

Examined again April 23rd, noon (about forty-eight hours from commencement of experiment). Took the control plate of organisms into a south room having direct strong sunlight from an open window. The organisms after a time collect *very* slightly to sun side, but in the quite open unshaded plate are fairly indifferent, being distributed all over.[1] Now one-half of the dish was shaded by cardboard, the line of shade, as on previous occasions, being arranged parallel to incidence of the light; at once all the organisms came out into the sunlit half, somewhat more at the point nearest to the sun. On reducing the sunlit part to a very small space, it became crowded with organisms accumulating more densely at the point nearest to sun. This compact group of organisms was pipetted off from the *white* plate into a *black* vulcanite half-plate photographic developing dish, containing sea-water, when the organisms, *at once almost*, accumulate at the part of the dish *farthest* from the sun. The half of the black dish farthest from the sun, after stirring up, was covered over (that is, with the line of shade at right angles to plane of incidence), and the organisms all collect, at remotest end of shaded part, away from sun.

This peculiar reversal in the black dish is difficult to explain, unless it was due to the absence of reflection. The result could not be repeated in other experiments because the organisms were never again found

1. This is a very exceptional behaviour in sunlight.

indifferent in sunlight, but always strongly negative, even in the white plates.

The organisms in this experiment were observed for four days longer; at the end of the third day they had become very strongly negative in the diffuse daylight of the north window, a reversion, it will be observed, from their original mixed condition with a large preponderance of positive organisms. While in this strongly negative condition they were taken into the dark room and tested with lamplight from a small oil lamp. At once there was a change; three of the five, viz., Nos. 3, 4, and 5, were now altered to positive, while Nos. 1 and 2 were mixed, partially positive and partially negative.

The plates were left in the dark room over-night, the only trace of illumination being a very faint ruby light, coming from a small borrowed light through the double thickness of a ruby window and ruby photographic screen.

The following morning, as soon as a light was struck in the dark-room, it was seen that all the organisms in all the plates were collected at the points nearest to the faint ruby light.

The small oil lamp was lit and the plates arranged round it; all five showed the organisms strongly *positive*. Taken out immediately from the lamplight to the diffuse daylight of the north window again, the organisms in all five are found to be strongly negative. No interval save the time of shifting the plates out from dark room to window bench elapsed between these two observations with reversed results.

Experiment II. Tow-netting taken by Professor Herdman, on April 23rd outside the Bay. On standing in diffuse daylight of north window, two large groups separate in the glass jar; as usual, one towards light and at top, the other away from light and at bottom of jar. These two groups were separated off by pipetting into two soup plates, one containing the positive group, the other the negative group, and both were found to consist chiefly of Nauplii of Balanus.

The negative group was taken first for examination in the dark room. On lighting one candle the organisms swim to the opposite pole; on placing two candles at opposite diameters of the plate, the organisms lie in the middle, half-way between the two lights; with four candles placed around equi-distant, the organisms are clustered compactly at the centre of the plate. The positive organisms similarly examined show a grouping around the periphery of the plate accentuated opposite each candle.

The organisms were left in the dark room overnight and examined in it next morning. On first striking a light, *both* sets of organisms were

seen clustered at nearest point of each plate to the exceedingly faint ruby light. One candle was lit and placed close to the plate containing the previously *negative* organisms, these are now nearly all *positive* to this intensity of light. Next morning (11 a.m.) both sets of organisms, which had remained in the dark room overnight, when tested by candle light were strongly positive. They were at once taken out of the dark-room and placed on the window bench in the north room in fairly strong diffuse daylight (it had been snowing, and the hill across the Bay from the Station was covered with snow). All the organisms in the originally negative plate were now negative again: those in the originally positive plate were mixed about three-fourths negative and the remainder positive.

The two plates were once more carried back to the dark room and tested to candle light. The originally negative plate, which a few minutes before had been completely positive in the dark room to candle light, had now, on account of its short sojourn in the diffuse daylight, turned to partially negative and partially positive in about equal groups. The originally positive group was still all positive to the candle light, although a few minutes previously in the diffuse light of the window about three-fourths of the organisms had been positive.

Three points are shown clearly in this experiment.

First, that the reaction varies *in the same organism at the same time* with the intensity of the light, and that feeble illumination gives a positive reaction and strong illumination a negative one.

Secondly, with the same intensity of illumination the reaction varies with the previous history and exposure to light of the organism. Exposure to darkness or feeble illumination turns the organism so that it reacts positively, and previous bright illumination changes it so that it reacts negatively to a strength of stimulus to which it before acted positively.

Thirdly, throughout these series of changes the original bias of the particular set of organisms persists, the other effects being superposed in a roughly algebraic summation. Thus the original trends towards positive and negative in the two sets of organisms dawn out again at the end of the experiment.

Experiment III.—On velocity of movement in light of varying intensity and colour.

This experiment on the velocity of movement in light of different intensity and of different colour, was made by observing the time required for the organisms to swim from one end to the other of a flat, black vulcanite dish of rectangular shape. The length of the dish was 17 cm.,

and the organisms were first brought to a compact mass at one side by placing the light to be used at one end and then the time noted for them to swim across and form a similar compact mass at the other end when the source of light was shifted to that end.

Then the time was again noted which they require to swim back to their original position; these times are denoted by 'Out' and 'Back' in the following table. In taking the time 'out' one does not wait for every organism, but waits till the great majority are in a compact group, this, after a little practice can be done accurately within a quarter of a minute or less.

For different intensities of light, one ordinary paraffin wax candle was used in one case, and four similar candles in the other case. For white light, the dish was simply uncovered, and for coloured lights it was covered completely over with slips of coloured glass, through which the coloured light passed to reach the organisms. The coloured glasses which it was possible to obtain were red, green and blue. Regarding the total intensity of light passing through the three slips, it appeared to the eye as if the red strip was most obscure, and the green most transparent, the blue being intermediate, but no exact photometric instrument was available. The organisms used were a strongly positive group obtained by pipetting off in diffuse daylight.

1. Illumination intensity = one candle.

Red light ...	Time 'Out'	... 3 min. 0 secs.
	Time 'Back'	... 3 min. 0 secs.
Blue light ...	Time 'Out'	... 3 min. 30 secs.
	Time 'Back'	... 3 min. 20 secs.
Green light ...	Time 'Out'	... 3 min. 40 secs.
	Time 'Back'	... 3 min. 0 secs.

2. Illumination intensity = 4 candles.

White light...	Time 'Out'	... 3 min. 0 secs.
	Time 'Back'	... 2 min. 50 secs.
Red light ...	Time 'Out'	... 3 min. 0 secs.
	Time 'Back'	... 3 min. 0 secs.

Experiment IV.- Selection of position under different coloured glasses with the same direction of incidence.

In this experiment, diffuse daylight was used on some occasions and candlelight on others, the long side of the dish being placed parallel to the surface of the window, or next to the candles. Then the two glasses of the two different colours to be compared were placed edge to edge, each covering

one-half of the dish, the edge where the two slips of glass touched being at right angles to plane of window, so that each half was situated exactly the same as to direction of incidence and intensity of light from the window; and a similar arrangement was used with the candlelight, the candles being so placed opposite the middle of one of the long sides of the dish that they shed equal light on the two different coloured halves. Before placing the two slips over the dish, the contents were stirred so as to uniformly distribute the organisms, but care was taken that the contents were not rotating when the slips were put over. Also, after the organisms had distributed themselves selectively, and the result had been noted, the two slips were reversed in position, each to each, and the change in distribution observed; the organisms at the time were strongly positive in the candle-light, and strongly negative in the diffuse daylight.

First, using four candles in the dark room, and with the red glass on the left-hand half and the blue glass on the right-hand half, in 2 min. 30 secs. from the commencement all the organisms are under the blue glass and next the candles, none under the red glass. The red and blue glasses are now reversed without disturbing candles or organisms, and in a very short time all the organisms have shifted and are once more under the blue glass in its new situation.

Second, similar results obtained with diffuse daylight, except that organisms now swim from the light; with blue and red most of the organisms under blue, a few only under red; with blue and green, two groups form at the two corners distal to the light, the larger of the two groups being under the green. Thus the organisms move with equal velocity under all coloured glasses, but when two colours are offered for selection they accumulate chiefly under one. Further, the direction of movement to pass from one colour to another is across the direction of incidence, and not to or from the light, and the relation to the light of the two halves being the same, it would appear that a preference for a particular colour or wavelength (or the greater or lesser stimulus of different wavelengths), caused the different distribution. If the organisms are carefully watched when they are becoming distributed, it is seen that they do not move directly across from one half to the other, but are moving about apparently freely, an organism every now and then leaving a group and darting off; but there is a certain amount of steering and controlling during these apparently free movements, which ultimately settles them down in their final distribution.

In this type of experiment, observation of the grouped animals shows, as in all the other experiments where the animals are grouped either

positively or negatively under the influence of the light, that there is no such thing as fixed and continuous orientation of the minute animals. In every group a great many are moving about in and out amongst one another, and a good many are entering and leaving the group like bees from a hive, but each individual, after a short trip about soon returns to the group. The source of light is, in fact, a strong directive influence, but there is no rigidly fixed orientation, any more than there is in a cluster of midges, or a brood of chickens around their mother.

Experiment V.—Movement in converging and in diverging light.

In order to obtain converging and diverging light, cylindrical museum jars, about 10·5 centimetres in diameter and 18 centimetres high, were used, which happened to be in stock at the Station.

Two such jars were used; the first, filled with clear fresh water, was used only as a cylindrical water lens, and contained none of the organisms; the second jar contained the organisms in sea-water. The first cylinder was placed a variable short distance, up to about one foot, from a small oil lamp with a circular wick, and the second cylinder was placed close up against it, on the other side from the lamp. The lamp and two cylinders were so arranged that the diverging light from the lamp became slightly convergent in passing through the first cylinder, and being still further converged by the second cylinder, it formed a caustic about two-thirds to three-fourths of the way through the second cylinder, and from that onward to the concave surface of the second cylinder the light was diverging.

By this arrangement any organism moving along the path of the rays, either towards or away from the light, is forced in one part of its path to travel in converging light, and the remaining part it travels in diverging light. Experiments were carried out both with white light and with coloured lights. The first set of organisms examined were negative; these swam away from the light into light of *increasing* intensity towards the caustic, and then through this onward in light of decreasing intensity till they reached the glass surface most remote from the light. Positive organisms were next tried, and swam in the exactly reverse direction, first from the most distal part towards the caustic in converging light, and therefore of increasing intensity, and then onward in diverging light, therefore of decreasing intensity, up to the glass surface nearest to the light.

At first sight it looks proven from this that intensity of light is of no effect, and the direction of incidence the whole matter, because the

organisms appear to swim in one direction indifferently, whether the illumination is increasing or decreasing. In reality, however, such a conclusion would be fallacious, for in order that, say, a *positive* organism should turn when it began to swim in light of gradually *decreasing* intensity, it would be necessary for it to turn its sentient surface away from the light, and that would plunge it into darkness.

The true conclusion is shown by what might be termed secondary effects seen on carefully watching the above experiment with negative organisms. These organisms at first accumulate in the narrow band of light at the distal glass surface from the light, where they dart about in small curves, keeping close to the glass; but in a few minutes it is found that a great many of them have accumulated in the two shady margins just outside this strongly illuminated band, and on either side of it. The probable explanation of this is that for these negative organisms the feebler light outside the band is nearer the optimal stimulus, and when they escape from the direct light beam in the course of their peregrinations, they find a suitable stimulus in the feebler light. But when any accident, such as a chance movement stimulated by some other cause, sends them again into the beam, they are stimulated to turn away from the light, and must again return *via* the distal glass surface to the refuge of the shade again.

This effect is seen still more strikingly when the red glass strip is interposed on the path of the incident light; then scarcely a single organism is seen on the illuminated strip, but two packed masses are seen on each side of it in the shade, and gradually tailing off as the distance from the illuminated strip increases. Similar results are seen with negative organisms if a narrow opaque white strip, such as a strip of cardboard, be lowered into the jar and held in a vertical position at the caustic. When the light is now placed in position, any organisms in the course of the beam, or swimming into it from the two dark zones at either side of it, turn at once away from the light, and swim along the path of the rays towards the caustic and the card; but they do not accumulate to any appreciable extent at the card, they swim round its edges and accumulate in the narrow feebly-lit space behind it.

Experiment VI.—With young larvae of the plaice (Pleuronectes platessa).

A number of young plaice larvae, which were five to seven days old, were taken from the Fish Hatchery attached to the Station, and placed in sea-water in a flat, oblong pie-dish. It was found that they were faintly

negatively phototactic in diffuse daylight. Contrary to the case of the Nauplii, this appeared to be increased in lamplight as well as in direct sunlight. When the dish is brought into lamplight in the dark room, it is found that most of the larvae after some time are accumulated in the half of the dish farthest from the lamp, decreasing to a clear space directly under the lamp. There is, however, no such tight packing up as in the case of the Nauplii.

The interesting point, however, is that there is no evidence whatever of orientation in regard to the light; the larvae lie at rest with their long axes at all possible angles with the line from the lamplight, some directly facing it, some straight away from it, others nearly at right angles, and many indiscriminately at all angles. The arrangement is not a chance one, as it looks at first sight, for no matter how often the larvae are disturbed and stirred up, they finally settle with the great majority in the distal half, and lying there at rest at all angles to the direction of incidence. On shading one-half of the dish with cardboard, the line of shade being parallel to the plane of incidence, the great majority of the larvae are found in the shaded half, more in the distal quadrant, and in all lines of orientation. If cards are arranged so that one quadrant of the dish only is illuminated, that quadrant becomes almost free.

Experiment VII.—Indifference of phosphorescent organisms to movement in light from without.

It was thought that organisms which themselves emitted light might show interesting results in their reactions to light from without, and this led to the work of Section B about to be described; but it was found that the phosphorescent organisms present, probably certain copepoda, were entirely indifferent to incident light, at any rate as far as movement was concerned.

Since the organisms could not be made to phosphoresce in the dark room during the day, the procedure was adopted of taking a tow-netting during the day, when the Bay was known, by observations made during the previous night, to contain abundance of phosphorescent organisms. This tow-netting was placed in diffuse daylight, and nearly all the positive organisms were pipetted off into one dish containing sea-water, nearly all the negative organisms were similarly pipetted into a separate dish, and finally, a good number of indifferent organisms were pipetted off into a third dish, from the middle of the bottom of the stock jar.

The three sets of organisms were then examined for phosphorescence after dark, when phosphorescence where organisms were present had

spontaneously set in and could be further intensified by stirring. It was then found that the positive and negative portions each contained only one or two phosphorescent organisms taken up unavoidably with the others; but the indifferent set contained a large number of phosphorescent organisms. The indifferent set containing the majority of the phosphorescent organisms were also practically indifferent to candle-light. In regard to numbers of organisms in each set, the positive set were by far the most numerous, and the numbers in the indifferent and negative sets were about equal.

The experiment was varied in a fresh tow-netting by placing several flat pie-dishes containing the organisms (not separated off on this occasion as to phototaxis) around the lamp in the photographic room, just after nightfall, until the usual phototactic groups had separated, then extinguishing the lamp, and watching the spontaneous appearance of phosphorescence without disturbing the dishes. There is no spontaneous phosphorescence for a period of about two minutes under such circumstances, then it commences, and it is seen that the phosphorescent organisms are scattered about indiscriminately in each dish, and not arranged in any relationship to where the light had previously been. Sometimes the phosphorescing organisms are moving about rapidly while illuminated, but in the majority of cases they are almost or quite at rest, and it is probable that if there had been any previous movement of a phototactic character while the lamp was lit, the arrangement would not have quite disappeared in the short interval after the light was extinguished before the spontaneous phosphorescence reappeared.

The only conclusion from the experiments appears to me to be that these particular phosphorescent organisms are almost or quite indifferent to incident light.

B.—Diurnal Periodicity in Phosphorescence

The suggestion of the work described in this section arose incidentally, as above-mentioned, and at the time the experiments were made it was unknown to me that a diurnal periodicity in phosphorescence had previously been observed and described.

A search through the earlier literature, however, revealed a description of its occurrence in Pyrophora by Aubert and R. Dubois,[1] and in Noctiluca by Massart.[2] Henneguy[3] states that Noctiluca does not

1. *Compt. rend. acad.*, T. XLIX, p. 477, 1884; *Compt. rend. soc. d. biol.*, p. 661, 1884. See also papers in both these Journals by R. Dubois, 1884-6.
2. *Bulletin scientifique de la france et de la belgique*, T. XXV, p. 72, 1893.
3. *Compt. rend. soc. d. biol.*, XL, p. 707, 1884.

light up until it has been kept in the dark for half an hour, and that the intensity is not at the maximum for another additional half-hour.

The following passage from Massart describes the variations as observed in Noctiluca:—

'The experiments show that the irritability is dependent on the alternations of day and night, the Noctiluca is hardly excitable on shaking during the day and shines only during the night. Fact still more curious, whether the organisms are submitted to the alternations of day and night, or whether they are maintained in constant illumination or constant obscurity, they still remain much more excitable during the night than during the day. It is a veritable phenomenon of memory, everything looks as if the Noctilucae preserved the recollection of the regular succession of the days and nights.'

Massart compares this to the change in position of the leaves of plants during day and night in the Oxalis and certain Papilionaceae, but adds that while the phenomenon lasts only some days in plants, in the Noctilucae it lasts until the death of the animal.

His experiments at the outside limit, however, lasted for one week only, when the organisms died; in the present set of observations the diurnal alternation of activity was followed with organisms kept in continuous darkness for twelve days, and although the number of living organisms was decreasing all the period, a few were still left alive and phosphorescent at night at the end of the period.

Since the fact of this diurnal periodicity is one of the most striking of those alternating habits or functions of the lower invertebrates which bear such a curious resemblance to memory in higher vertebrates, and, indeed, have been regarded as a rudimentary memory,[1] it may be regarded as sufficiently interesting to merit a detailed description. It appears to stand in some danger of being forgotten, since it is not mentioned even in the larger of the modern text-books, and to the best of my knowledge it has not been shown to exist in the phosphorescent copepoda, nor demonstrated as persisting for such a long period as in the present experiments. Also its onset at the close of the day and gradual extinction at dawn have not previously been followed with any exactitude.

1. See F. Darwin, Presidential Address, Brit. Association, Dublin, 1908.

DIARY OF EXPERIMENTS

Monday, September 21st, 1908 (8-30 p.m.) Calm night, and sea very phosphorescent. Collected plant (*Polysiphonia nigrescens*) from the rope of an old mooring buoy. The plant is covered over with phosphorescent organisms which flash most brilliantly. The specimen is preserved in sea-water and examined ashore. It shows most brilliant phosphorescence when rubbed. When a piece is put in fresh tap water in the dark it lights up most brilliantly all over for about three minutes, then gradually the light fades out, and cannot now be evoked by any process of shaking or rubbing.

Tuesday, September 22nd.—The plant was taken into the dark room at 11 a.m. and examined; no phosphorescence could now be evoked by any process, either shaking in air, stirring up in the sea-water, rubbing, or applying fresh water.

A tow-netting had just been taken in the Bay (12 noon). This was taken into the dark room at once, but no trace of phosphorescence could be obtained from it, even with most vigorous stirring.

In the evening, from 9 to 9-30 p.m., a tow-netting was taken in the Bay, the sea being very phosphorescent wherever touched by the oars. The haul, when taken into the boat, scintillated most brilliantly while being washed into sea-water in a jar. The contents of the jar, taken into the dark room at the Station, are showing spontaneous phosphorescence, and give a vivid show when stirred. Left in the dark room over-night.

Wednesday, September 23rd.—Examined the previous night's tow-netting at 11 a.m.; there is not a trace of phosphorescence to be elicited, even on stirring briskly. Examined at intervals all day in the dark room. There is not a trace of phosphorescence seen till about 6-30 p.m., when sparking first starts on stirring, just as it is growing dusk outside, and at 7 p.m. there is spontaneous phosphorescence.

Took also during the day three tow-nettings from a row-boat, each of 15 minutes' duration, at 12-45 to 1 p.m., 3-45 to 4 p.m., and 5-15 to 5-30 p.m. As each tow-netting was finished, it was taken to the Station, at once emptied into a flat pie-dish, and taken to the dark room to be examined for phosphorescence. On each such occasion the tow-nettings previously there were also examined, as also at other intervals during the day. In none of the three was any phosphorescence seen till about 5-40 p.m., when a single organism was seen to spark in the second tow-netting (taken 3-45 to 4 p.m.), but nothing in the first or third.

Examined at 6-45, when it is dusk outside, all three are

phosphorescing spontaneously, bright sparks showing up, sometimes three or four at once in each dish. On stirring there is a bright display lighting up each dish. All three left over-night in dark room.

Thursday, September 24th.—Examined at 10 a.m., none of the three tow-nettings show any phosphorescence in the dark room. Nos. 1 and 3 were kept in the dark room all day, while No. 2 was kept in the daylight, but taken at intervals to the dark room for examination. No phosphorescence seen in any of the three at any time during the day; but at night (7 p.m.) all three are sparking spontaneously, showing bright sparks at intervals. The phosphorescence is increased on stirring, so that six to ten phosphorescent spots are visible at once, but the display is not so brilliant as on the previous night, probably owing to deaths.

All three left in dark room till Friday morning; the faint ruby light from the dark room window is completely shut off by banking it up with cardboard on the outside.

This same day, being a bright day with good sunlight, three additional tow-nettings were taken, at 11 to 11-15 a.m., 12-45 to 1 p.m., and 4-45 to 5 p.m., and examined in future along with the other three, being kept in dark room also. Examined as follows in dark room:—

No. 1 observed at 11-30 a.m.	... ...	No phosphorescence.
Nos. 1 and 2 observed at 1-15 p.m.	... ...	No phosphorescence.
Nos. 1, 2 and 3 observed at 5-10 p.m.	...	No. 1, Nil; No. 2, single spark on vigorous stirring; No. 3, Nil.
Nos. 1, 2 and 3 observed at 5-35 p.m. (Good light outside).	...	Nil; single spark; Nil.
Nos. 1, 2 and 3 observed at 6-35 p.m. (Almost dark outside).	...	Spontaneous sparking in all three, No. 3 most brilliant. Dish lit up in each case on stirring.
Nos. 1 2 and 3 observed at 6-50 p.m. (Quite dark outside).	..	All spontaneously phosphorescing most brilliantly.

Also at 1 p.m. to-day, a further supply of *Polysiphonia nigrescens* was collected from the old mooring rope, and examined in the dark room. It showed no phosphorescence during the day. On placing in distilled water it gives a feeble sparkling, but incomparably less brilliant than on

similar treatment at night. After dark, from 6-30 p.m. onwards, the same sample sparkles when stirred, and a piece put in distilled water lights up brilliantly all over for from three to five minutes; then the light dies away, and cannot further be evoked in that piece by any of the procedures mentioned.

All six of the tow-nettings of yesterday and to-day examined again at 7-15 p.m.; all spontaneously phosphorescing, and showing up brilliantly on stirring. Same result when again examined at 8-40 p.m.

Friday, September 25th.—Arrived at Biological Station at 4-50 a.m; there is just a trace of dawn in the dull, grey sky. Organisms examined at once in the dark room, where they have all still been kept over-night; all six dishes are flashing spontaneously.

On standing quietly by and watching the phosphorescence, the minute organisms are not moving about rapidly in most cases, and one can observe that each active organism is emitting a series of flashes at about the rate of one per minute, and between the flashes there is a dimmer light showing which regularly becomes increased by a flash. The effect on the eye is very similar to that of a revolving light seen at sea at some distance off. There is an almost constant dim light lit up by repeated and fairly regular flashes.

Many of the more active organisms are so still that one is able to observe clusters of four or five in nearly constant positions for some minutes, so as to give an impression of constancy of shape to the group for the time resembling a stellar constellation.

The effect in the complete darkness of the dark room is very beautiful as the undisturbed organisms spontaneously flash out in the darkness.

The organisms were now observed at frequent intervals of about ten minutes, in order to accurately note the decline and disappearance of the phosphorescence. It was observed that the number of organisms flashing out was decreasing all the time. The rate of decrease became very rapid about 5-30 a.m., when the daylight was just beginning to grow rapidly brighter outside. At 6 a.m. there was only an occasional odd flash in each dish, showing that only a few organisms in each were still active. At 6-15 a.m. only one dish (the third of those collected on Thursday) was still showing an occasional gleam; all the other five dishes had stopped spontaneous phosphorescence. At 6-30 a.m. all spontaneous phosphorescence had disappeared, but a faint display could still be elicited in all six dishes by vigorous stirring. At 7 a.m. no sparking obtainable in any dish, even on most vigorous stirring; same result repeated at 7-30 a.m.

The organisms on the *Polysiphonia nigrescens* behave similarly to the free organisms in the dishes throughout. It was feared that the organisms would perish if the sea-water were not changed, so Nos. 1 and 2 of the Wednesday tow-nettings were filtered in the dark room through the silk of the tow-netting, and then the net being turned (so that no fresh organisms could be introduced), the organisms were washed into a fresh quantity of sea-water poured on to the net. Hence there were in future five dishes to observe instead of six, but no alteration in rate of survival on account of the changing was observed, and, as the other dishes of organisms appeared to be doing well, the process of washing into a fresh supply of sea-water, which was exceedingly difficult and awkward in the quite dark room, was abandoned.

The organisms were next examined at 1 p.m., when vigorous stirring failed to call forth a single spark in any of the tow-nettings or on the weed.

The next examination was at 9 p.m., when every one of the dishes showed spontaneous phosphorescence. The display in the three Thursday nettings is not so vivid as on the previous night, there being fewer organisms phosphorescing. It is also noticeable that the phosphorescence is not so vigorous in each individual organism. The flare out is perhaps as great, but the light completely dies out in all cases after each flare, and the period between the flares seems to be lengthened, so that one cannot pick out a particular organism by its flashes and keep track of it. The two dishes from the Wednesday tow-nettings, which are to-night showing for the third time, are not much decreased in vigour from the second night, either in frequency of spontaneous flashing or in vividness on stirring them. Nearly as many phosphorescing organisms appear to be present, and the flashes are about as bright as on the preceding night.[1]

These Wednesday organisms have now lit up for the third time, having been quite quiescent in the intermediate periods of daylight in the outer world. One of the two dishes has been in complete darkness throughout the period. From this onward all the sets of organisms are kept in complete darkness the whole time.

Saturday, September 26th. The organisms were examined at 11 a.m., and again at 1 p.m., when no sparking was occurring, nor could any be evoked by vigorous stirring. The next observation was commenced at 6-07 p.m., when the daylight was commencing to fade outside. The dishes were not stirred, but quietly watched in the complete darkness. When the first spontaneous flash occurred, the dark room was quitted and the time

1. This was observed in nearly all the experiments, a great drop during the first twenty-four hours, and then a very slow death-rate in the residue

noted; it was 6-13 p.m. Between 6-15 and 6-30, six flashes were counted; between 6-30 and 6-45, twenty-two flashes; between 6-55 and 7-15 p.m., there were twenty flashes. The display is much less marked than on the previous evenings. On stirring the dishes, three or four organisms can be made to phosphoresce at once in each case.

The organisms on the *Polysiphonia nigrescens* are also phosphorescent on stirring.

Sunday, September 27th.—Examined at 10-30 a.m.; no phosphorescence, either spontaneous or on stirring, from any of the dishes. Re-examined at 7 p.m., four of the dishes show spontaneous phosphorescence, the rate of sparking being extremely slow. The remaining dish (the third of the Thursday tow-nettings) has undergone putrefaction, and shows no phosphorescence, even on stirring. It is taken from the dark room, and all the organisms in it are seen to be dead.

Stirring elicits two to four phosphorescent organisms at the same time in the remaining four dishes.

Monday, September 28th.—Examined the four dishes at 2-30 p.m.; no phosphorescence obtainable from any of them. Examined again at 7-30 p.m. Two spontaneous sparks seen in the Wednesday dishes in an interval of about five minutes; no spontaneous phosphorescence seen in the Thursday dishes. On stirring, about six phosphorescent organisms seen in one of the Wednesday dishes, and three or four in the other; one seen in the first of the Thursday dishes, and three or four in the second.

Tuesday, September 29th.—Examined at 3-30 p.m.; no phosphorescence visible or obtainable. Examined again at 9 p.m., there is spontaneous phosphorescence in both of the Wednesday dishes, and in one there is an organism which remains steadily phosphorescent with a dull glow all the time. On stirring, about six phosphorescent organisms are visible in each of the Wednesday dishes, and the sparking is brilliant. In the Thursday dishes, on stirring, there is less display, only two or three organisms showing up at once in either. The few organisms are, however, quite active, and a single organism in each case lights up so as to illuminate the contents and sides of the whole dish.

Wednesday, September 30th.—Examined at 11 a.m.; no phosphorescence, spontaneous or otherwise. Examined again at 7-30 p.m., no spontaneous phosphorescence during a period of about 5 minutes, but on stirring there is a good display in all four dishes. This is the eighth night of appearance of phosphorescence in the Wednesday lots, and seventh night for the Thursday organisms.

Thursday, October 1st.—Examined the four dishes at 11 a.m.; no

phosphorescence, spontaneous or on stirring. Examined again at 7 p.m., there is spontaneous phosphorescence at a slow rate in three (two Wednesday and one Thursday), and in all four on stirring.

Friday, October 2nd.—Examined at 3 p.m.; no phosphorescence, either spontaneous or on stirring. Examined again at 9 p.m.; in one of the Wednesday dishes there is an organism which remains permanently lit up the whole time of observation, about seven minutes. Spontaneous phosphorescence seen in the other Wednesday dish, and in one of the Thursday dishes. All four give phosphorescence on stirring.

This is the second time a continuously phosphorescent organism has been observed. It may be a pathological condition of the organism.

Saturday, October 3rd.—Examined at 4 p.m., no phosphorescence of any kind; did not examine after nightfall this day.

Sunday, October 4th.—Examined at 12 noon, no phosphorescence in any dish, either spontaneously or after vigorous stirring. Examined again at 6-20 p.m., and watched at intervals till 8-30 p.m., but there is no spontaneous flashing. On stirring, however, there is phosphorescence obtainable in each of the four dishes, one or two organisms only flashing in each case.

The experiments were brought to an end at this date. When the dishes are taken to the light it is found that only a small number of organisms are visible and alive in each, and there is much débris of dead organisms.

The diurnal periodicity of the phosphorescence had been observed for twelve days and nights in the case of the organisms collected on Wednesday, September 23rd, and for eleven periods in the case of those collected on Thursday, September 24th, without any exception. During this interval, with the exception of one of the Wednesday dishes which had been exposed to light on the first Thursday of the period, all the dishes were kept in continuous darkness, yet at the close of the day the organisms always lit up, and lights were extinguished about daylight in the morning.

The four dishes of organisms were now filtered one after the other into the small end of the same tow-net, washed out into a little sea-water, and fixed with five per cent. formol.

The fixation was carried out in the dark room in order to observe if there was any phosphorescence. About six bright points shone out, two of which persisted brilliantly for about three minutes, and then faded out.

The weed (*Polysiphonia nigrescens*) was kept in the dark from the Thursday (September 24th) till Wednesday (September 30th), showing

phosphorescence at night and none during the day. Fearing that it would decompose, it was then placed in ordinary diffuse daylight in a vessel with running sea-water. This treatment increased the amount of phosphorescence enormously, and in a day or two it was quite as phosphorescent as at first. Taken from the diffuse daylight to the dark room for examination, it was never phosphorescent, but at night it always phosphoresced most brilliantly. It, also, at the end, was fixed in 5 per cent. formol, and in this process lit up about twenty seconds after the application of the formol, and shone vividly for about three minutes before dying out.

The examination of the united tow-nettings was difficult on account of the majority of the organisms being dead and in a broken-up condition through the long duration of the experiment, but the following account was kindly given me by Mr. A. Scott, to whom my best thanks are due:—

DIATOMS.—*Biddulphia mobiliensis*, 1,000; *Chaetoceros densum*, 50; *Coscinodiscus radiatus*, 50; *Trochisca sp.*, 250.

COPEPODS.—*Calanus helgolandicus*, 20; *Pseudocalanus elongatus*, 680; *Temora longicornis*, 100; *Centropages hamatus*, 10; *Paracalanus parvus*, 100; *Isias clavipes*, 100; *Copepod nauplii*, 100; *Copepod Juv.*, 200.

MOLLUSCA (larval).—Gasteropods, 150; Lamellibranchs, 500.

No Noctilucae were present.

It is not probable that the diatoms or molluscan larvae were phosphorescent, so that there is little doubt that the phosphorescence was due to the copepods present, or certain species of these.

The following is a statement of the contents of the routine tow-nettings always taken of the plankton of the Bay, for the statistical work of the Biological Station, on the date (Thursday, September 24th) when the second set of tow-nettings were collected for the observations:—

DIATOMS.—*Biddulphia mobiliensis*, 800; *Chaetoceros decipiens*, 600; *Ch. densum*, 440; *Coscinodiscus radiatus*, 50; *Streptotheca thamensis*, 150; *Trochisca sp.*, 50.

DINOFLAGELLATA, &c.—*Ceratium furca*, 50; *C. fusus*, 100; *C. tripos*, 100; *Tintinnopsis sp.*, 600.

COPEPODA.—*Calanus helgolandicus*, 50; *Pseudocalanus elongatus*, 3,180; *Temora longicornis*, 280; *Centropages hamatus*, 65; *Acartia clausi*, 2,200; *Oithona similis*, 1,750; *Paracalanus parvus*, 830; *Isias clavipes*, 160; *Copepod nauplii*, 3,960; *Copepod juv.*, 2,180.

MOLLUSCA, &c.—Lamellibranch larvae, 280; Oikopleura, 3,800.

Whether this diurnal periodicity has the same physical basis in a rudimentary fashion as memory in higher animals, is still an open question, for it is open to believe that the alternating play of light and darkness upon those cells which produce the phosphorescence may have induced in them a periodicity of activity and rest which still persists after the alternating stimulus is withdrawn. The process may, for example, be due to a secretion by certain cells which phosphoresces as each drop is produced, and this process of secretion may have a period of rest during the day and activity during the night. The rhythm of this activity may be timed daily under ordinary conditions, and regulated by alternation of light and darkness. During the day there would be storage in the cell, and at night discharge. On the removal of the stimulus of light during the day this state of alternation of rest and action might persist for a long period.

CONCLUSIONS

1. The characters of the response of an organism by movement to light are not constant for a given organism, but vary for the same organism at the same time according to the intensity of the light and the previous history of the organism in regard to light. As a general rule, the organism is positive to feeble light and negative to stronger light, and for a constant intensity of light at a given moment previous darkness or weak stimulation tends to turn organisms positive, and previous exposure to bright light turns them negative.

2. Both the positive and negative behaviour to light may be explained on the basis of one chemical action of light upon the cell (a katabolic one). The positive state indicates that the speed of reactions in the cell lies below a certain value, which may be called the optimal value, and the negative state corresponds to a speed of reactions in the cell above the optimal value. In the former case the sentient surfaces are turned into the light to increase velocity of reaction up towards the optimal value; in the latter case the sentient surfaces are turned away

from the light so as to decrease the velocity of the reactions down towards the optimal value.

3. As a result of the orientation so caused, there arises movement of the organism towards or away from the source of light, but such orientation is not a fixed orientation, but rather a steering action; the animals as a result do not remain in one fixed plane or direction of movement, but the net result of the movement is that the organisms move to or from the light. When the movement is finished the organisms (plaice) may lie in all possible planes of orientation to the light.

4. Movement towards or away from the light has in some organisms (Nauplii of Balanus) an associated movement upwards or downwards. These two movements would coincide together in natural movements of the organisms under the influence of light alone in the sea.

5. In the case of Nauplii of Balanus, addition of small amounts of acid or alkali was not found to alter the reactions to light.

6. The rate of movement of the organism (Nauplii) is almost the same with different intensities of light and different coloured lights, showing that the locomotor apparatus is not affected by the light, but continues to work at the same rate.

7. The particular organism used (Nauplius of Balanus) moves from red light to blue light, and from blue to green, under such circumstances that the incident light is the same in direction for both coloured regions. This would indicate that with the particular total intensities being used for the experiments, green is a more suitable or optimal stimulus than blue, and blue in turn more optimal than red.

8. Movement in converging and diverging light is described and shown to be explicable on the basis of intensity of light alone, and that direction produces its effects in a secondary manner on account of the light and shade effects of the animal's own body.

9. The phosphorescent organisms experimented with (certain copepods) were shown to be indifferent, in regard to movement, to light from without.

10. That light from without has another type of influence upon these phosphorescent organisms is shown, however, by the fact that their periods of activity and rest in regard to phosphorescence follow respectively the hours of daylight and darkness.

11. It is shown that this alternating diurnal periodicity can persist for a long period (twelve days) in absence of the accustomed recurring stimulus of the light and darkness of day and night.

12. The phosphorescence of these copepods in captivity is

spontaneous, and although increased by mechanical stimulation, it goes on vigorously even when the organisms are undisturbed and quite still.

13. When the organisms are freshly taken, the character of the phosphorescence is such that a faint light persists, which is increased at intervals by bright flares or flashes. At a later period the light disappears entirely between the flashes, which have a longer interval between them. Under probably pathological conditions, after the organisms have been kept confined for a considerable period, there may be lighting up of the organisms with a continuous glow.

14. The appearance of the spontaneous phosphorescence at nightfall, and its disappearance at dawn, are characterised by the same changes in a reversed order in the two cases. Before the appearance of spontaneous phosphorescence at night, and after its disappearance in the morning, there is a period of minimal excitability of about half an hour during which stirring still calls out phosphorescence. After this the organisms become completely refractory.

15. Addition of fresh water, or formol, produces, during the period in which the organism is dying, a most vivid phosphorescence, which lasts from two to three minutes, and then fades and disappears.

This display is very feeble during a daylight period, compared to what is seen after dark when spontaneous phosphorescence is present.

THE RELATIVE IMPORTANCE OF INORGANIC KATIONS, ESPECIALLY THOSE OF SODIUM AND CALCIUM, IN THE CAUSATION OF GOUT AND PRODUCTION OF GOUTY DEPOSITS[1]

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As long ago as 1844, Ure[2] suggested that calcium salts and sodium salts were deposited in gouty conditions: the sodium as biurate in the synovial membranes and tendons, and the calcium as phosphate in the arterial walls.

From the predominance of sodium salts in the fluids of the body it is to be expected that the bulk of any salt should contain that kation either in solution or as a deposit; but the more insoluble any salt-forming anion is, such as that of uric acid, the more important does the presence not of one but of several kations become; for upon such multiplicity of kations does the carrying power of the solvent for the feebly soluble anion depend.

The great importance of the relative effects upon one another of these kations, in common solution with uric acid, in dissolving or precipitating the uric acid anion, has not been sufficiently realised, and the solubilities under such conditions have not been sufficiently investigated.

The object of the present paper is to supply some further information on this question of solubility and precipitation of uric acid anion as acid salt, from the common solution containing more than one kation.

The various salts of calcium and uric acid have been described by Delepine[3] at length, as well as their occurrence in urine and tissues. He describes (1) an acid salt (biurate) which is comparatively soluble in water, and (2) a basic or neutral salt (normal urate) comparatively insoluble. He then quite justly remarks that this is a reversal of what obtains in the case of alkaline urates, presumably those of sodium and potassium, and calls attention to the evident importance of this in relation to the reaction of the solvent medium.

Following Heintz, Delepine and other authors, quote calcium biurate as a highly soluble biurate, being even more soluble than the potassium biurate.

1. Part of the expense of this research has been defrayed by a grant from the British Medical Association.

2. *Medical Times*, Vol. XI, p. 145, 1844.

3. *Journ. of Physiol.*, Vol. VIII, 1887.

Thus Neubauer and Vogel give the following figures for solubility of different biurates in water in the cold and at boiling point:—

Lithium biurate,	1	part dissolves in		370	parts cold, and	39	parts boiling water.		
Calcium	,,	1	,,	,,	603	,,	276	,,	,,
Potassium	,,	1	,,	,,	790	,,	75	,,	,,
Sodium	,,	1	,,	,,	1,150	,,	112	,,	,,
Magnesium	,,	1	,,	,,	3,750	,,	160	,,	,,

The fact that calcium biurate occurs in all tophi, as shown by Ebstein and Sprague,[1] and usually to the extent of 12 to 13 per cent, alongside of about 57 per cent. of sodium biurate, is sufficient to indicate that the statements and figures given above as to the solubility of calcium biurate, do not represent the true state of affairs accompanying deposition in the body, which must occur at body temperature, and from media which, being rich in sodium chloride, may behave quite differently as a solvent from distilled water.

It is peculiar that no experiments have been recorded as to the solubility at body temperature, and from salt solutions. Such experiments I have undertaken, and have found, first, that calcium biurate must be placed even lower than magnesium biurate at the bottom of the scale of solubility of biurates, when the solubility is measured in distilled water kept at body temperature in a thermostat; secondly, that mere traces of a calcium salt added to solutions of sodium biurate causes precipitation; and thirdly, that the presence of so little as 0·5 per cent. of sodium chloride enormously lowers the solubility of sodium biurate.

These facts taken together appear to me to explain why sodium biurate and calcium biurate appear together in tophi, and give a significance to calcium biurate in gout, similar to that shown by O. T. Williams[2] for the insoluble calcium soaps secreted by the intestinal mucosa in mucous colitis and appendicitis, and by Klotz and others for the calcium saponification in arterio-sclerosis, where the insoluble soap sooner or later passes into the form of the likewise insoluble calcium carbonate.

The biurates used for our experiments were prepared from Merck's 'extra pure' uric acid. In the case of the calcium and magnesium biurates, the composition was controlled by incineration and weighing the calcium oxide and magnesium oxide respectively. The calcium salt yielded 12 per cent. of CaO, theory requiring in the biurate 13·6 per cent.,

1. *Ergebnisse der Physiologie*, Bd. II, 1903.
2. This Journal, Vol. II, p. 395, 1907; Vol. III, p. 391, 1908.

and in the normal urate 27 per cent. Similarly, the magnesium salt yielded 11·7 per cent. of MgO, the theoretical yield being 11·2 per cent. for biurate and 21 per cent. for normal urate. It is, therefore, obvious that in each case we were dealing with the biurate.

The results obtained for solubility in distilled water of the four biurates were as follows:—

One part of potassium biurate dissolves in	64	parts boiling water and in	550	parts at body temperature
" sodium " "	117	" "	1,030	" "
" magnesium " "	148	" "	2,440	" "
" calcium " "	666	" "	4,760	" "

The last figures are the average of two independent titrations with potassium permanganate, which were carried out most carefully, and the result corroborates the findings of those observers who give calcium biurate a permanent place amongst the constituents of tophi. If it were so soluble as Delepine, and the other authors whom he quotes, would place it, then it could not be present in such gouty deposits.

In the body, however, the various biurates are not dissolved in distilled water, but in a saline solution containing as the chief constituent sodium chloride, along with other inorganic salts in lesser concentrations. Accordingly an attempt was instituted to approach more closely to natural conditions by determining the solubilities of the above biurates in a half per cent. solution of sodium chloride.

Here the most interesting result was obtained that while the solubilities of calcium and magnesium biurates were actually somewhat increased in the saline solutions, that of the sodium biurate was reduced almost to zero.

This result gives probably the key to two things, first, that in the body the preponderating salt in gouty concretions is sodium biurate, and secondly, on account of the increased solubility of the biurates of the alkiline earths (Ca and Mg) in salines, we see, perhaps, a rational basis for understanding the improving effects of certain saline mineral waters empirically used in gout.

In any case, the variation in the figures according to whether the solvent medium for the biurates is water or a dilute sodium chloride solution, gives an indication of the great value which would attach to the study of the solvent action of solutions containing a number of inorganic salts in varying proportion.

Attention may be specially called to the small amount of sodium chloride which produces such a considerable change in the solubilities, illustrating that small variations in relative distribution of the salts of the

plasma, such as might naturally occur from individual to individual, may have profound effects on the solubilities of the biurates in the body.

The enormous effects of the sodium chloride on the solubility of the sodium biurate is, in the language of physical chemistry, probably to be referred to the mass action of the common sodium ion of the sodium chloride and sodium biurate, tending to throw out of solution the less soluble of the two salts, viz., the sodium biurate. At least, such an action has certainly been shown to occur in countless cases in pure solution of salts possessing a common ion in their constitution. Hopkins and Hope[1] quote Nernst's generalization to the effect that any two salts susceptible of dissociation, which contain an electric ion in common, naturally diminish each other's solubility. But the converse of this proposition has been found to hold true in certain cases, and salts possessing no electric ion in common may mutually increase each other's solubility in a fluid. The possibility exists, therefore, that the ingestion of a mixed dietary may produce such a temporary increase in the proportion of salts other than those of sodium (especially potassium salts) as to increase the solubility of any retained sodium biurate, and so accelerate its excretion. They consider this an important principle in lithiasis.

For ease of comparison the results of titration of the saturated solutions of the four different biurates, (*a*) in distilled water, and (*b*) in half per cent. sodium chloride solution, in each case at body temperature, are given in the following table. The figures show c.c. of $\frac{n}{20}$ $KMnO_4$, required to oxidize the uric acid, and to get quantities of uric acid dissolved these must be multiplied by the factor 0·00375. In the second part of the table this has been done and the results re-stated in terms of the amount of fluid in each case required to dissolve one part of uric acid in the form of the respective biurates at body temperature.

Biurate taken	Amount dissolved by 100 c.c. of distilled water at body temperature as uric acid	Amount dissolved by 100 c.c. of 0·5 per cent sodium chloride solution at body temperature
Calcium biurate	5·7	7·8
Sodium "	**25·9**	**0·6**
Magnesium "	11·0	13·6
Potassium "	48·4	48·0

Biurate taken	Parts of distilled water required to dissolve one part	Parts of 0·5 per cent. sodium chloride required to dissolve one part
Calcium biurate	4,760	3,420
Sodium "	**1,030**	**44,400**
Magnesium "	2,440	1,960
Potassium "	550	555

1. *Journal of Physiology*, 1898-99, p. 284.

Precipitating Effects of Calcium Salts on Sodium Biurate Solutions

Another important relationship is the precipitating effect of calcium salts on biurate solutions of sodium. It was found that the addition of even small percentages of calcium salts to distilled water had the effect of very much lessening its solvent power on sodium biurate.

Experiment I.—Calcium chloride—

At 37° C.	100 c.c.	distilled water alone in 12 hours		took up an equivalent of		25·9 c.c.	$\frac{n}{20}$ $KMnO_4$
,,	100 c.c.	,,	+ 0·1 % $CaCl_2$	,,	,,	3·5 c.c.	,,
,,	100 c.c.	,,	+ 0·3 % $CaCl_2$	,,	,,	1·08 c.c.	,,
,,	100 c.c.	,,	+ 0·5 % $CaCl_2$	,,	,,	1·9 c.c.	,,

So that calcium chloride exerts a decidedly deterrent influence on the solubility of sodium biurate in aqueous solution.

Experiment II.—Calcium sulphate :—

Control in distilled water			25·9 c.c.	$\frac{n}{20}$ $KMnO_4$
,,	,,	+ 0·1 % $CaSO_4$	19·0 c.c.	,,
,,	,,	+ 0·2 % $CaSO_4$	15·0 c.c.	,,

Calcium phosphate also had a decided effect in lowering the solubility, and this has a special interest in being so frequently found in tophi.

Experiment III.—Calcium Phosphate.—The following titrations give detailed quantitative results to show the markedly deterrent effect of small percentages of calcium phosphate on the biurates in aqueous solution.

Three solutions containing distilled water (*a*) with potassium, and (*b*) sodium biurates in excess, and (*c*) a mixture of the two in excess were placed in the incubator at 37° C. To three other samples of these same solutions ½ per cent. of calcium phosphate was added, and these were similarly treated. Three titrations were made with the following results per 100 c.c. :—

I.

(*a*) Distilled water with KHU in excess after 2 hours at 37° C.
had dissolved an equivalent of 63 c.c. $\frac{n}{20}$ $KMnO_4$

(*b*)	,, ,,	NaHU	,, ,, ,,	34 c.c. ,,
(*c*)	,, ,,	both biurates	,, ,, ,,	75 c.c. ,,

II.

(*a*) Distilled water with both biurates in excess after 72 hours at 37° C.
had dissolved an equivalent of 60 c.c. $\frac{n}{20}$ $KMnO_4$

(*b*)	,, ,, ,, ,, ,, ,,	38 c.c. ,,
(*c*)	,, ,, ,, ,, ,, ,,	75·5 c.c. ,,

III.

(*a*) + ½ % $CaHPO_4$ in excess after 72 hours at 37° C. had dissolved
an equivalent of ... ... 46·5 c.c. $\frac{n}{20}$ $KMnO_4$

(*b*)	,, ,, ,, ,, ,,	27·5 c.c. ,,
(*c*)	,, ,, ,, ,, ,,	54·0 c.c. ,,

These results derive added importance from the fact that $CaHPO_4$ is constantly found in tophi, and emphasises the value of recent research in demonstrating the excretory function of the intestine for calcium and for phosphates. They also go towards giving a rational explanation for the empirical treatment by mercurials, salines, etc., which prevailed in the past, and is still recommended, for constipation is a classical symptom in gout (Sydenham).

Complementary to the general question of solubility comes that of the formation of deposits and tophi, and the influence of calcium salts in this direction. There is a presumption that its influence is decided, for we have already pointed out that calcium is usually present as a biurate or a phosphate.

Without wishing to exaggerate the value of experiments *in vitro*, as applied to body processes, one is tempted to suggest some analogy between an experiment like the following and the conditions prior to an acute attack of gout, for, as Osler states, 'the formation of tophi must rest upon some physico-chemical basis of precipitation and crystallization.'

This experiment is intended to demonstrate the powerful precipitating effect of a calcium salt in minute percentages on a solution of sodium biurate containing amounts varying from 1 in 819 to 1 in 3,275, which Roberts believed to approximate to the amounts possibly occurring in a supersaturated condition of the blood and sera preceding an acute attack.

A solution was made of uric acid (Merck's extra pure) in a ·2 per cent. sodium of bicarbonate of sodium. This was found by direct titration with $\frac{n}{20}$ $KMnO_4$ to require 36 c.c. per 100 c.c. of solution, corresponding to a strength of 1 in 655 of uric acid. Into eight stoppered bottles, the following quantities of this solution were poured, 80, 70, 60, 50, 40, 30, 20, respectively, and distilled water added to bring each volume up to 100 c.c. These represent strengths of biurate of sodium varying from 1 in 819 to 1 in 3,275. To each was added a weighed amount of crystallized sulphate of sodium, equivalent to ·2 per cent. of the anhydrous salt. The bottles were then placed in the incubator at 37°C., and left for twenty-four hours. At the end of that time they were still clear. Another ·1 per cent. of anhydrous sulphate was added, and as at the end of three hours they were still found to be clear, ·1 per cent. acid phosphate of soda was added.[1] Three hours afterwards they were still clear, and they remained so during the next twenty-four hours. At the end

1. These salts were employed so as to minimize the error in the final titration due to oxidizing of the chloride.

of that time ·01 per cent. $CaCl_2$ was dropped from a stronger solution, and after sixteen hours they were found to be still clear. At the end of that time another ·01 per cent. $CaCl_2$ was added. This produced no immediate effect, but after *one* hour slight flocculi were visible in certain of the bottles. Ten c.c. of each solution (previously filtered) gave the following results on titration, which show clearly the precipitating effect of $CaCl_2$ even in ·02 per cent.

				Milligrammes found	Milligrammes theoretical	% loss of Uric acid
(1)	80 c.c. solution	+ 20 c.c. water	+ 0·28 Na_2SO_4 (0·45 Na_2SO_4 10 H_2O) + 0·2 % NaH_2PO_4 + 0·02 $CaCl_2$	2·1	2·9	27·6
(2)	70 c.c. ,,	+ 30 c.c.	,, ,, ,,	2·0	2·4	16·4
(3)	60 c.c. ,,	+ 40 c.c.	,, ,, ,,	1·9	2·2	13·6
(4)	50 c.c. ,,	+ 50 c.c.	,, ,, ,,	1·7	1·8	5·5
(5)	40 c.c. ,,	+ 60 c.c.	,, ,, ,,	1·3	1·4	7·1
(6)	30 c.c. ,,	+ 70 c.c.	,, ,, ,,	1·0	1·08	7·0
(7)	20 c.c. ,,	+ 80 c.c.	,, ,, ,,	0·7	(0·72)	2·8

Regarding the precipitating effect of sodium chloride in gout, and the use of common salt in the diet, the statements in the literature are somewhat contradictory. Sir W. Roberts[1] advised abstinence from the use of much culinary salt in the gouty. He believed it could be stored up, especially in the serous fluids, to a concentration sufficient to impede solubility, and presumably by inference cause precipitation. Hofmeister's work (quoted in Schaefer's Physiology) tends to corroborate this suggestion by proving the power for adsorption of sodium chloride which colloids of the chondrin and mucin type possess. Roberts himself quotes figures in his published Croonian lectures to show that a maximum sodium content is found in cartilaginous tissues.

Mendelssohn[2] mentions experiments with some of the well-known uric acid solvents. He dissolved piperazin and lysidin in blood serum and 'found such solutions to have as great a solvent power for uric acid as their aqueous solutions. If sodium chloride be added precipitation occurs, and in the form of sodium biurate.' He continues: 'The sudden nature of attacks of gout seems to point to sudden formation of some such urate precipitant, and the fact that they generally occur after mistakes in diet, over-feeding, etc., where much salt is consumed seems to indicate sodium chloride to be the cause.'

Dapper and von Noorden, in a monograph recently published on the effect of sodium chloride on metabolism, discuss the effect of sodium chloride waters upon the excretion of uric acid. They find in therapeutic literature the greatest confusion on the point as to whether it increases or diminishes the output of uric acid in gout. They cite quantitative

1. *Croonian Lectures*, 1882.
2. *Deutsch. med. Wochensch.*, 1895, p. 283.

results from several cases to prove that such saline waters do increase the output of uric acid, and state that these facts point to the use of saline mineral waters in our treatment of gout as worthy of consideration.

Two cases in my own practice have afforded suggestive evidence that sodium chloride has something to do with gouty-joint phenomena. In the first of these cases a child of five years developed acute arthritic symptoms in the feet and thighs, simulating acute rheumatism, after an overdose of sodium chloride administered as a home cure for intestinal worms. Previously, too, a fairly strong salt solution had been injected per rectum daily for the same purpose. That the sodium chloride had some pathogenic significance was proved by the fact that as soon as its administration ceased, with no other medication except small doses of potassium citrate, the child became rapidly well and has been well ever since.

The second case occurred in a gardener (with an alcoholic bias) who had been enjoying a course of boiled ham. He had suffered from podalgic gout before, and this now again developed itself first in one great toe joint and then in the other.

In this case two samples of 24-hours' urine (150 c.c.'s in volume) were analysed for the quantitative estimation of the bases, (1) one when the attack was at its worst, (2) the second when recovery was well advanced, at an interval of several days. The exact amount of chlorides was not tabulated. Sodium chloride, however, was found in excess, but in regard to the bases, the figures are—

	Na_2O	K_2O	CaO	MgO	
(1)	2·041 grs.	0·091 grs.	0·067 grs.	0·085 grs.	in 150 c.c.
(2)	1·375 grs.	0·183 grs.	0·122 grs.	0·093 grs.	of urine

This, so far as it goes, would suggest that sodium chloride in the acute stage had something to do with arthritic symptoms, and that an increase of urinary calcium, magnesium, and potassium are at least compatible with recovery from an attack of gout, whilst the reverse is true for sodium.

Whether sodium chloride can exist in such concentration as to cause precipitation or not, it is much more likely that a metal of higher valency than sodium, especially one such as calcium, which can produce a higher insoluble biurate, will cause precipitation.

It may be noticed also that a gouty condition is often associated with lead poisoning, as Garrod pointed out in his early researches.

In conclusion I have to express my thanks to Dr. Charles E. Harris for valuable assistance in carrying out the solubility experiments.

ON THE NITROGEN-CONTAINING RADICLE OF LECITHIN AND OTHER PHOSPHATIDES

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Part I

Since the investigations of Diaconow and Strecker[1] it has generally been assumed that lecithin is a compound of fatty acids with glycero-phosphoric acid and a base choline. This assumption is based on the results of elementary analysis combined with the fact that hydrolytic decomposition of the lecithin yields the above-mentioned constituents. From this it is obvious that the total amount of nitrogen present is represented by the nitrogen of the choline radicle, and in this way a knowledge of the total amount of nitrogen yielded by any pure lecithin makes it easy to deduce the amount of choline ($C_5H_{15}NO_2$) actually present, from a theoretical standpoint. Many experiments have been made in order to obtain the choline content of different lecithins, but in every case the results actually obtained fell far below the theoretical values. Thus Erlandsen[2] obtained from pure heart lecithin, which had been split up by boiling with barium hydrate, only about 42 per cent. of the theoretical amount, and Heffter,[3] using lecithin extracted from liver, obtained under similar conditions only 25 per cent.

In order, if possible, to elicit the cause of these losses, the following investigation was undertaken; here a comparison of the amounts of choline actually obtained from different lecithins, saponified and manipulated under exactly similar conditions, suggests much as to the real nature and cause of this loss in certain lecithins.

Material Used

For the first set of experiments a lecithin sold by the firm of J. D. Riedel, Berlin, under the trade name of 'lecithol' was employed. Afterwards, I extracted and purified lecithin from the heart muscle of the ox, as described below.

1. *Annal. d. Chem. u. Pharm.*, 148.
2. *Zeits. f. Physiol. Chem.*, Bd. LI, S. 113.
3. *Arch. f. exp., Pathol. u. Pharm.*, Bd. XXVIII, S. 100.

'Lecithol'

At first the lecithin salt of cadmium chloride was made use of, the lecithin being dissolved in alcohol, and the solution carefully filtered, and then precipitated by cadmium chloride. This lecithin salt, however, while much more convenient to weigh and handle than lecithin itself, gave rise to much difficulty on attempting to split it up with solution of barium hydrate, either in water or alcohol; here it constantly adhered to the sides of the flask above the liquid, so that, even by constant shaking, it was difficult to ensure complete saponification. For this reason it was discarded, and the lecithin itself used. The lecithin was found, however, to contain traces of ammonium compounds, and in order to get rid of these impurities it was treated as follows. Small quantities were gradually added to some water in a mortar and ground up until a completely homogeneous emulsion was formed. This emulsion was precipitated by means of acetone, the lecithin carefully filtered, kneaded into a plastic mass with some more acetone, and again emulsified as before. The filtrate gave abundant evidence of the presence of ammonia when treated with caustic alkali and heated. After the process had been repeated three times it was found that acetone failed to give a precipitate. This difficulty was overcome by the addition of a few drops of sodium chloride solution, when the lecithin separated out quite readily. This process of emulsification and precipitation by acetone was carried out five times, the last two filtrates giving no indication of the presence of ammonia. The lecithin was then thoroughly washed with acetone, and dried in vacuo over sulphuric acid. After drying, it was dissolved in absolute alcohol, and the solution preserved in a well-stoppered dark bottle. For each experiment 5 c.cm. of this solution was used, the same pipette, each time thoroughly cleaned and dried, being used for measuring. This lecithin solution gave the following results on analysis:—

Nitrogen (Kjeldahl's Method)

In four experiments 5 c.c. solution = 10·2 10·0 10·0 and 10·2 c.c. $\frac{n}{10}$ H_2SO_4

Average 10·1 c.cm. = 14·14 mgrs. N

Phosphorus (Neumann's Method)

In five experiments 5 c.c. solution = 52·0, 52·5, 51·9, 52·1, 52·3, c.c. $\frac{n}{2}$ NaOH

Average 52·2 c.cm. = 28·89 mgr. P

Ratio of P : N = 1 : 1·08

∴ 5 c.c. alcoholic sol. = 14·14 mgrs. N = 0·31073 grm. cholin platinum chloride.

In another solution used—

5 c.c. = 17·4062 mgr. N = 0·38250 grm. cholin platinum chloride.

EXPERIMENTS WITH 'LECITHOL'

Five c.c. of the above alcoholic solution was taken and added to 100 c.c. of methyl alcohol, saturated with barium hydrate, and boiled in a flask on the water bath for varying periods; the flask, which was fitted with a reflux condenser, was shaken from time to time. After heating for periods of one to ten hours,the mixture was allowed to stand for some time, when a well-marked precipitate separated out and fell to the bottom of the flask, the fluid above remaining fairly clear. This fluid was filtered off and the precipitate returned to the flask; to the flask was added 100 c.c. of ethyl alcohol, and after being well shaken up with the residue was boiled for five minutes. The mixture was then allowed to stand and the clear alcohol filtered off as before. This process was repeated usually about four times, and sometimes oftener, in order to ensure thorough extraction of any choline remaining in the insoluble residue. All these alcoholic extracts were then mixed together and carefully evaporated on the steam bath to about the bulk of the original amount of alcohol (100 c.c.) or sometimes rather less. The barium was then separated by treatment with hydrochloric acid; the barium chloride was filtered off and the fluid evaporated to dryness. In order to avoid losses from bumping, it was found better to perform all these evaporations in flasks.

When carried out in this way it was easy to ensure that nothing was lost. The dried residue was then thoroughly extracted with absolute alcohol, evaporated to small bulk and treated with a saturated solution of sublimate in absolute alcohol. This was left to stand till next day when the precipitate was separated by filtration; the filtrate was then evaporated to dryness, and the residue, after being washed with cold alcohol, added to the first precipitate; the combined precipitates were then dissolved in hot water. This solution was treated with sulphuretted hydrogen and filtered; filtrate was evaporated to dryness and dissolved in a little absolute alcohol. The choline, which was present in the form of choline chloride, was now precipitated by a solution of platinum chloride in absolute alcohol; after eighteen to twenty-four hours the precipitate was filtered off, washed with cold absolute alcohol, dried and weighed.

Sublimate was introduced on the assumption that possibly the presence of impurities might interfere with the precipitation of the choline by platinum chloride, and that those impurities might to a greater extent be got rid of by sublimate. Subsequent results did not bear out this view, and in all my later experiments sublimate was omitted.

An extended series of observations with the above method has been already published[1] by the writer, but the following short extract serves to show the general results obtained. In fifteen experiments the average percentage of the lecithin nitrogen obtained as cholin nitrogen was only 77·3 per cent:—

Number	No. of hours hydrolysed	Choline-platinum-chloride: Actual amount found, in grms.	Theoretical amount, calculated from N of lecithin, in grms.	Percentage actually found, in terms of theoretical amount
1	1	0·2700	0·38250	70·59
2	1½	0·2450	0·31073	78·85
3	2	0·2941	0·38250	76·89
4	4	0·2991	0·38250	78·19
5	7	0·2421	0·31073	77·91
6	10	0·2390	0·31073	76·91

That the substance obtained was pure choline platinum chloride is evident from the following figures:—

1. 0·2439 grm. left on ignition 0·0771 grm. Platinum = 31·61 %
2. 0·1740 grm. „ 0·0550 grm. „ = 31·61 %

Calculated for $(C_5H_{14}NOCl)_2\,PtCl_4$ = 31·64 % Pt.

In three experiments, carried out exactly as above, only that sublimate was not used, the following results were obtained. Here, also, the salt proved to be pure, giving on ignition a residue of 31·59 per cent. Pt.

Number	No. of hours hydrolysed	Choline-platinum-chloride: Actual amount found, in grms.	Theoretical amount, calculated from N of lecithin, in grms.	Percentage actually found, in terms of theoretical amount
1	2	0·2976	0·38250	77·80
2	3	0·3003	0·38250	78·51
3	3	0·3031	0·38250	79·24

In all these experiments it is seen that not more than from 77 per cent. to 79 per cent. of the total nitrogen can be recovered as choline nitrogen.

Here it is interesting to note that at the same time as these results were published, a paper appeared by Moruzzi[2] describing the results of hydrolytic decomposition by sulphuric acid. In his experiments the double salt of cadmium-chloride-lecithin was utilised. The following figures taken from his paper show practically the same percentage of

1. *Zeits. f. physiol. Chemie*, Bd. LV, S. 363.
2. *Loc. cit.*, S. 352.

choline platinum chloride (average 77·7 per cent.) as was obtained by the writer with barium hydrate.

Number	Amount of cadmium compound used, in grms.	No. of hours hydrolysed	Choline-platinum-chloride: Actual amount found, in grms.	Choline-platinum-chloride: Theoretical amount, calculated from N of lecithin, in grms.	Percentage actually found, in terms of theoretical amount
1	2·8450	4½	0·6642	0·8687	76·5
2	1·4952	4½	0·3507	0·4574	76·7
3	1·8147	4½	0·4425	0·5542	79·8

Hydrolysis in Watery Solution of Barium Hydrate

Some experiments were now made in order to observe what results could be obtained by using a saturated watery solution of barium hydrate instead of an alcoholic fluid. Five c.c. of the lecithol solution was added to 100 c.c. of a saturated solution of barium hydrate in water, and boiled with a reflux condenser for 2½ hours; during the process the flask was shaken from time to time, especially during the first hour. It was then allowed to stand, and the precipitate filtered off. After thorough washing of the residue, the filtrate was freed from barium by means of CO_2; barium carbonate was filtered off, and the filtrate, after the addition of a little hydrochloric acid, evaporated to dryness. Residue was extracted with a little absolute alcohol, and after evaporating to small bulk, was precipitated directly with platinum chloride. Precipitate was then left to stand till next day, filtered, washed, dried and weighed as usual. Here the average result obtained was 77·5 per cent.

In all the above modifications it is seen that the result remains practically the same, so that not more than about 77 per cent. to 79 per cent. of the theoretically calculated choline-platinum-chloride is actually obtainable by experiment from this particular lecithin.

The following table indicates the average results of the different methods:—

		Percentage of Choline-platinum-chloride obtained, calculated on theoretical amount
1. Lecithin hydrolysed with saturated solution of barium hydrate in methyl alcohol, using sublimate as intermediate precipitant	=	77·3 %
2. Same as above, without sublimate	=	78·5 %
3. Saturated watery solution of barium hydrate	=	77·5 %
4. Sulphuric acid (Moruzzi)	=	77·7 %
Average	=	77·75 %

On the Causes of this Loss of the Theoretically Calculated Choline

Here we have to deal with a loss of a little over 20 per cent. of the theory, and in order to elicit its cause the following points were considered:—

(1) Is the choline partly destroyed when heated with a saturated solution of barium hydrate?

Since it is known that the *free* base decomposes on heating into trimethylamine, ethylene oxide, and H_2O, and since, as first observed by Heffter, a mixture of lecithin and barium hydrate which has been boiled for some time has a pronounced smell of trimethylamine, it might be thought that this decomposition was the cause of the loss. Gulewitsch,[1] however, has shown that this destruction of choline is so small as to be of little practical importance, and some experiments made by the writer entirely supported this statement. Lecithin was boiled with barium hydrate in a flask fitted with a reflux condenser, the latter being connected with a bottle containing $\frac{n}{10}$ H_2SO_4. Through this acid the volatile decomposition products of lecithin were led by means of a stream of ammonia-free air, and the amount of acid neutralised estimated by titration. In various experiments lasting from two to eight hours it was found that the amount of $\frac{n}{10}$ H_2SO_4 used was very small, amounting, after long boiling to not more than the equivalent of 1 to 3 per cent. of the total nitrogen of the lecithin used. From this it is obvious that the loss is not accounted for, or at least only to a very small degree, by the decomposition of the choline.

(2) Is the lecithin completely split up, and does the filtrate contain the total nitrogen of the lecithin used?

That the lecithin is completely split up would appear from the fact that boiling for ten hours gives no better result than boiling for two to three hours. As the result of experiment, we may assume that all the lecithin is entirely split up after two to three hours or even less. With regard to the N-content of the filtrate, it is interesting to note, that in no case was it found to contain all the nitrogen of the saponified lecithin. An analysis of the residue after boiling also showed that this residue always contained nitrogen in a form insoluble in alcohol, as no amount of washing had any effect in lowering the amount of this residual

1. *Zeits. f. physiol. Chemie*, Bd. XXIV, S. 513.

nitrogen. This residue was always very carefully washed in the following manner: after filtration it was returned to the flask and thoroughly shaken up with about 100 c.c. alcohol: the mixture was then boiled for five to ten minutes, allowed to stand, and again filtered. This process was generally repeated three times, but in some experiments as often as six times: in each case the result was the same. The number of hours during which the mixture was boiled had also no effect on the result. The average amount of insoluble nitrogen found in the residue amounted, as shown by the following table, to 8·5 per cent. of the total nitrogen of the lecithin.

No.	Number of hours boiled	Total N of lecithin used, in mgrs.	N found in residue, in mgrs.	Percentage of total N found in residue
1	1	14·14	1·19	8·4
2	1	14·14	1·13	8·0
3	1½	14·14	0·98	7·0
4	1½	14·14	1·26	8·9
5	3	14·14	1·13	8·0
6	3	14·14	1·19	8·4
7	5	14·14	1·33	9·4
8	5	14·14	1·26	8·9
9	7	14·14	1·26	8·9
10	7	14·14	1·33	9·4
	Average	14·14	1·21	8·5

(3) Does the possible presence of traces of impurities (other decomposition products of lecithin) prevent the complete precipitation of choline by platinum chloride?

To test this, some pure choline chloride solution (0·1 to 0·2 per cent.) in absolute alcohol was taken and divided into equal parts by means of a burette. To some of these portions were added glycerophosphoric acid, glycerine and barium chloride; they were precipitated with platinum chloride. The other portions were directly precipitated. A comparison of the results obtained in both cases showed that the presence of these impurities, while tending to lower the percentage of choline-platinum-chloride obtained, did not do so to any marked degree. Considering that only very minute traces of impurities can be present, it is not likely that this factor is of much importance practically, in preventing complete precipitation.

(4) Is choline chloride imperfectly precipitated by platinum chloride in alcoholic solution?

Gulewitsch made an experiment bearing on this point in the following manner. He took a 0·5 per cent. solution of choline chloride in absolute

alcohol and precipitated it with an alcoholic solution of platinum chloride. After twenty-four hours the precipitate was filtered off and washed with absolute alcohol. The filtrate was evaporated to dryness, after being decomposed by sulphuretted hydrogen gas. The residue, which was very small, gave only a slight cloudiness with phosphotungstic acid and with iodine and potassium iodide solution. From this it was concluded that platinum chloride precipitates choline chloride quantitatively. Since, however, the quantities obtained in lecithin experiments are necessarily small, it was thought advisable to weigh some choline-platinum-chloride salt; then dissolve it in H_2O, decompose the solution with sulphuretted hydrogen, evaporate the filtrate to dryness, dissolve out with absolute alcohol and precipitate with platinum chloride. The amount of the latter salt ultimately obtained was compared with the amount used. In all my experiments it was found that the weight of choline salt obtained in this way always fell somewhat short of the original amount used. While it is likely that there must be some slight mechanical loss on account of the necessary manipulation, it would seem that the precipitation is not quite complete, and in this way a certain amount of the loss of the theoretical choline of lecithin is explained. In some experiments carried out with crystals that had been several times re-crystallised, and using alcohol that had been treated with barium oxide immediately before use, I obtained on an average from 93 to 97 per cent. of the original salt after the above manipulations. The average percentage, when using alcohol that had not been so treated, was somewhat lower. With care this loss is but slight.

The chief losses, therefore, seem due to part of the nitrogen remaining in the residue, together with small losses represented by the necessary manipulations of the method, combined with the incomplete precipitation of choline chloride by platinum chloride.

When we subtract the nitrogen found in the residue from the total lecithin nitrogen, we get theoretically the amount present in the filtrate; practically, however, owing to the necessary manipulation, it is obvious that the ultimate filtrate, when freed from impurities and ready for precipitation by platinum chloride must contain somewhat less nitrogen.

The *actual* amount of choline platinum chloride found, when reckoned on the nitrogen of the lecithin minus the residue nitrogen, was from 86 to 87 per cent. The *relative* amount of platinum salt obtained when the filtrate was treated as described on page (55), was from 91 to 92 per cent. of the total filtrate nitrogen. This difference as mentioned, is accounted for by small losses during manipulation, for

it is obvious that a comparatively small loss materially influences the percentage.

Since, therefore, platinum chloride tends to give incomplete precipitation of choline chloride, and since there may be traces of impurities also present tending to hinder precipitation, and when we consider the difficulty of absolutely exact estimation of the nitrogen in these experiments, it would seem that in this particular lecithin all, or nearly all, the nitrogen of the filtrate is present as choline.

Whether the nitrogen found in the residue is derived from the choline is at present undecided; it will be seen later on that a similar residual nitrogen is found after saponification of pure heart lecithin.

Lecithin from Heart Muscle

In order to compare the choline content of the lecithin of heart muscle with that obtained from Riedel's 'lecithol,' I prepared a pure lecithin in the following manner, much in the same way as Erlandsen has described.[1] At the same time the mono-amino-diphosphatide substance *Cuorin* was separated, as well as another substance behaving somewhat like a phosphatide and similar in physical qualities to the substance isolated by Stern and Thierfelder[2] from egg yolk, and designated by them 'weisse substanz.'

Preparation of Heart Muscle

Oxen hearts were procured as soon as possible after the animals had been slaughtered, and the fat and fibrous tissue separated off; the muscular substance was then cut up into small pieces and passed through a mincing machine. This finely divided material was spread out in a thin layer on a glass plate, and dried at 30°C. in a current of air, generated by a fan arrangement which was worked by a small motor. From time to time this layer was stirred, and the lumpy parts broken up into small pieces, and after twelve to eighteen hours it was generally quite dry. In order to obtain a fine powder suitable for extraction, this fairly friable dried material was broken down with the hand, and finally passed through a coffee mill. In this way a very fine powder was obtained. This was put into a desiccator and preserved in vacuo over H_2SO_4 until quite dry.

1. *Zeits. f. physiol. Chem.*, Bd. LI, S. 87.
2. *Loc. cit.*, Bd. LIII, S. 370.

Extraction

About 500 grms. of dried heart substance were taken, and to this about 1,000 c.c. ether added: this was put into a dark, well-stoppered bottle and shaken for two to three hours in a rocking machine; before shaking, the air in the upper part of the bottle was displaced by CO_2, in order to prevent as far as possible any tendency there might be for the lecithin to oxidise. After thorough shaking it was allowed to stand till next day and then filtered. In order to exclude oxygen during filtration, this process was carried out under an atmosphere of CO_2. A porcelain filter was carefully fitted into a glass bottle connected with a suction pump, and over this filter a large glass funnel was placed in an inverted position; this funnei was connected by a rubber tube to a cylinder containing CO_2, and during filtration a stream of this gas was allowed to pass so that oxygen was totally excluded. At first the ethereal extract filtered fairly well, but after some time it became very slow indeed. The filtrate was evaporated to about 150 c.c., and precipitated by excess of acetone. In order that the dissolved substance should separate out as well as possible it was found best to connect the dish containing the ether-acetone mixture with an exhaust pump for half an hour or so; the partial evaporation of the liquid generated sufficient cold to give a fairly complete precipitate. The fluid part was then poured off, and the solid substance obtained kneaded together with a fresh portion of acetone. This plastic mass was then dried in vacuo over H_2SO_4.

Each portion was extracted as above described five times; after this it was found that acetone yielded only traces of a precipitate. All the substance obtained in this way from the different extracts was mixed together and thoroughly dried. This substance, which represents the ether soluble part of heart phosphatides, contained, as above mentioned, pure 'lecithin' as well as other lecithin-like substances 'Cuorin' and 'white substance'; impurities such as fat and cholesterin were also present.

Separation of 'White Substance,' Fat, Etc.

Above mixture of substances was dissolved in ether in a dark well-stoppered bottle, and gave a whitish turbid fluid; it was not expected that a clear solution would be obtained, for the first 10 to 20 c.c. fluid that passed during filtration described above was invariably slightly turbid. This fluid was now centrifuged, when a great deal of a whitish substance was separated. The clear ethereal solution was evaporated to about

100 c.c., and then precipitated by excess of acetone, and the precipitate treated with a little fresh acetone and kneaded together into a plastic mass as before. This precipitate was mostly composed of dark brown masses, but part was also white and flocculent: these flocculent masses did not fall well, and could not easily be completely separated from the acetone along with the rest of the precipitate. When as much as possible was separated, the ether-acetone fluid still contained some flocculent masses. This mixture was left to stand over night in a closed vessel, being protected from light and air. Next morning it was invariably found that all the white masses were precipitated on the floor of the vessel, and were in all respects similar to the ordinary brownish precipitate first obtained, so that the apparent difference seemed to be only a physical one. This portion was then added to the first precipitate and dried as usual. The dried mass was then dissolved in ether as before, and the process again repeated. This was done until all the white substance was got rid of: at the same time fat and other impurities were separated. As will be seen from the following table it was necessary to centrifuge six times:—

No. of times centrifuged	Amount of ether used in c.c.	Nature of solution	After Centrifuging	
			Nature of solution	Relative amount of 'white substance'
1	250	Very turbid and of whitish appearance	Deep yellow-brown solution	Much 'white substance' From $\frac{1}{4}$-$\frac{1}{2}$ in. deep in tube of 1 in. diameter
2	250	Fairly turbid but much less so than in No. 1	Reddish brown solution	About $\frac{1}{2}$ $\frac{3}{4}$ amount of white substance in No. 1
3	200	Turbid, not markedly so	Light reddish brown solution	About $\frac{1}{4}$-$\frac{1}{2}$ amount found in No. 1
4	200	Slightly turbid	,,	Trace only
5	160	Slightly turbid	,,	Fair amount: about three times as much as in No. 4
6	160	Almost clear solution	Very light reddish brown solution	Slight trace of white substance after long centrifuging

Ethereal solution was evaporated each time to about 100 c.c. and precipitated with acetone. After above treatment the substance was thoroughly dried in vacuo over H_2SO_4.

The first ether extracts contained a fair amount of fat, but the last two portions seemed to be quite free from it.

SEPARATION OF SUBSTANCE INTO 'LECITHIN' AND 'CUORIN'

A substance was now obtained from which the 'white substance' was separated, and which was free from fat and cholesterin. This substance which gave a practically (though not absolutely) clear solution[1] in ether, contained 'lecithin' which is soluble in alcohol and in ether, and another phosphatide 'Cuorin' which is insoluble in alcohol. These substances were now separated as follows:—

The dried mass was dissolved in 150 c.c. ether and to this was added 600 c.c. absolute alcohol; the mixture was then placed in the ice cupboard for twenty hours. At once a certain amount of precipitate fell, but much more was evident after some hours, and next day the insoluble part (A) had apparently completely separated; the fluid was then filtered off, and the filtrate, which was of a light reddish colour, was partly evaporated directly under the pump and then placed in a desiccator in vacuo over H_2SO_4, and evaporated to dryness. When quite dry it was re-dissolved in absolute alcohol; here a small portion remained undissolved, and this was added to the precipitate first obtained. The alcoholic solution was then evaporated to small bulk, precipitated with acetone and dried as usual. It was completely soluble in ether, and appeared on drying as yellowish orange coloured masses (B).

In this way the substance was divided into two parts:

(A) Part insoluble in alcohol = 'Cuorin' (impure, containing some 'white substance').

(B) Part soluble in ether and in alcohol = 'Lecithin' proper.

ALCOHOL-INSOLUBLE PORTION (A)

The part insoluble in alcohol was thoroughly dried in vacuo and then dissolved in 150 c.c. ether. Solution was not clear, so centrifuge was used, and here again a good deal of white substance was separated off. The clear solution was precipitated with about four times its volume of absolute alcohol, and left to stand over night under CO_2 in ice. Next day the alcoholic solution appeared almost colourless. The precipitate obtained consisted of a mixture of white flocculent masses and a resinous syrupy material. This precipitate was treated with alcohol and left to stand in the incubator at 60° C. for several hours. The white part was now dissolved by the warm alcohol, and a dark brown syrup was left. This precipitate was again treated as above and a resinous mass again obtained = 'Cuorin.' On cooling the alcoholic solution, the white flocculent masses separated out and were filtered off = Portion A[1].

1. On centrifuging part of this solution no precipitate was obtained.

Purification of Cuorin

The Cuorin was dissolved in a little ether and gave a fairly but not quite clear solution; CO_2 was introduced, and after standing in the ice cupboard till morning a perfectly clear solution was obtained; a small amount of sediment was seen on the floor and sides of the glass. Acetone was now added, but the precipitate did not at first fall well, and an emulsion-like fluid was obtained. This was left standing in ice under CO_2 for sixteen hours; a good precipitate was now obtained as a brownish sticky mass. Acetone-ether mixture was slightly whitish coloured and when centrifuged gave a small precipitate of a brownish substance which was not added to the other portion. After centrifuging, the fluid was absolutely water clear. The precipitate, after being dried over H_2SO_4, was treated with warm ethyl acetate freshly distilled, and gave a clear solution; CO_2 was now introduced, and fluid was left to stand in ice for some hours, when precipitate settled down as a resinous mass on the floor of the flask. The ethyl acetate was somewhat light yellow coloured, and after separating it from the precipitate, was left to stand in ice till morning; then a little precipitate was found to have formed on the floor of the glass consisting of minute small white balls; this was thrown away. The precipitate was dried and the process repeated: this time the ethyl acetate after standing overnight remained quite clear. The substance was now dried, and a small amount dissolved in ether to test its solubility, when it was found that the solution was not absolutely clear. The whole precipitate was then dissolved in ether and left to stand under CO_2 till morning; fluid was now quite clear, and a little sediment appeared on the glass. This fluid was then evaporated to dryness and Cuorin obtained which gave quite a clear solution in ether.

Substance Soluble in Alcohol at 60° C. (A_1 Portion)

This, as already mentioned, was obtained when the hot alcoholic solution was allowed to cool; it was precipitated in white flocculent masses and only a small quantity was present; it was filtered off, and re-dissolved in alcohol at 60° C.; then allowed to stand under CO_2 in ice, and re-precipitated; it was then filtered, washed with cold absolute alcohol and dried over H_2SO_4 in vacuo. This substance seems to be the same as the 'white substance' separated previously by the centrifuge, but this point is at present being investigated.

The following plan shows roughly the general outline of the process of separation of the different phosphatides from ethereal extract:

Ethereal extract + alcohol =	precipitate (A)	treated with alcohol at 60° C.	*Portion* (A_1): dissolves at 60° and falls out on cooling: white substance
			Portion (A_2): Part undissolved = Cuorin
'Lecithin' remains in solution (B)			

Thus we have separated: (1) Lecithin;

(2) Cuorin;

(3) 'White substance' (by centrifuge);

(4) and part. soluble in alcohol at 60°, which seems to be 'white substance.'

Analysis of Heart Muscle Lecithin

In order to test the purity of this lecithin the following analysis was made. Phosphorus was estimated according to Neumann's method, and nitrogen after Kjeldahl.

Nitrogen

1.	0·5232 grm. Substance	= 6·9 c.c. $\frac{n}{10}$ H_2SO_4	=	1·84 %
2.	0·4134 ,,	= 5·5 ,,	=	1·86 %
		Average	=	1·85 %

Phosphorus

1.	0·3872 grm. Substance	= 27·22 c.c. $\frac{n}{2}$ NaOH	=	3·9 %
2.	0·2918 ,,	= 21·37 ,,	=	4·05 %
		Average	=	3·975 %

N : P = 1 : 1·03

C and H

1.	0·1747 grm. =	0·4234 grm. CO_2	=	66·12 % C
	and	0·1641 grm. H_2O	=	10·44 % H
2.	0·1925 grm. =	0·4688 grm. CO_2	=	66·41 % C
	and	0·1784 grm. H_2O	=	10·20 % H

	1	2	3	4	5	6	Average	Average of Eslandsen's analysis
C	66·12	66·41	—	—	—	—	66·27	66·29
H	10·44	10·20	—	—	—	—	10·32	10·17
N	—	—	1·84	1·86	—	—	1·85	1·87
P	—	—	—	—	3·9	4·05	3·975	3·95

The above result corresponds well with that of Erlandsen, and the results of analysis combined with its physical qualities show that this substance is a pure lecithin.

Saponification of Heart Lecithin

With Methyl Alcohol. This lecithin was now split up in order to compare the choline content with that of Riedel's lecithin. A carefully weighed quantity (from 0·3 grm. to 0·7 grm.) was ground up in a mortar

with 5 grms. solid barium hydrate. This was added to 100 c.c. methyl alcohol, and the mixture boiled in a flask fitted with a reflux condenser for periods varying generally from two to four hours, but sometimes as long as ten hours. Flask was then allowed to stand for some time when the fluid above appeared quite clear, and a well-marked sediment separated out on the floor of the glass. This clear fluid was carefully poured off and filtered, and from 80 to 100 c.c. alcohol added to the flask containing the residue; this was boiled for five minutes, left to stand and filtered as before. This process was usually repeated three times, and afterwards the residue was poured on to the filter paper, and washed there for some time. On some occasions, instead of pouring the residue on to the filter paper, it was thoroughly ground up in a mortar with some alcohol, and the boiling process repeated as above; thus the residue was sometimes boiled in all six times. The total filtrates were mixed and evaporated to about 100 c.c., and the barium precipitated by concentrated HCl; the mixture was carefully heated, allowed to cool and filtered from barium salts. The filtrate was evaporated to dryness and dissolved in water; this was filtered and filtrate again evaporated to dryness. Residue was now dissolved in a little absolute alcohol, and after being evaporated to small bulk was precipitated with platinum chloride and allowed to stand over night; precipitate was then washed, dried and weighed as usual.

Several slight modifications of the above method were from time to time introduced, but they did not in any way alter the results, and the above seemed, on the whole, the best.

Here the amount of choline-platinum-chloride obtained was only, on an average, *about 41 per cent. of the theoretical amount* when the substance was boiled for two to four hours. In some experiments in which boiling was carried out for five to ten hours the results were somewhat lower, the average being between 37 per cent. and 38 per cent. In no case was I able to obtain a higher percentage than 42·2. The following figures, quoted from a series, show generally the results obtained:

Number	Amount of lecethin used, in grms.	No. of hours boiled	Choline-platinum-chloride: Found, in grms.	Calculated from lecithin N, in grms.	Percentage of theoretical amount found
1	0·5231	2	0·0879	0·2127	41·3
2	0·4875	2	0·0792	0·1982	40·0
3	0·5916	3	0·1015	0·2405	42·2
4	0·4757	3	0·0810	0·1934	42·0
				Average	= 41·4

A mixture of above platinum salts yielded on ignition 31·9 per cent. Pt.

Residue after Saponification with $Ba(OH)_2$

This residue, after being thoroughly washed as above described, was now examined for nitrogen. As in 'lecithol' it was found that this residue invariably contained a certain amount of alcohol insoluble nitrogen. The following table gives the amount found in ten different experiments; the number of times that the substance was washed did not seem to have any appreciable effect on the nitrogen content. That this residual nitrogen was not present as unchanged lecithin is obvious from the fact that the lecithin would have dissolved in the alcohol; also the number of hours during which the lecithin was boiled made little difference in the result. The nitrogen was calculated by Kjeldahl's method, a blind experiment being made with the chemicals in each case.

No.	No. of times washed with 100 c.c. alcohol; boiled for 5 mins. each time	No. of c.c.'s $\frac{n}{10}$ H_2SO_4 used by residue	No. of mgrs. of nitrogen in residue	No. of mgrs. of nitrogen in lecithin used (average 1·85 % nitrogen content)	Percentage of total N in residue
1	3	0·44	0·616	9·68	6·4
2	4	0·39	0·546	9·01	6·0
3	3	0·45	0·630	10·94	5·8
4	1	0·35	0·490	8·8	5·6
5	2	0·49	0·686	11·50	5·9
6	5	0·57	0·798	15·37	5·2
7	3	0·39	0·546	14·09	3·9
8	3	0·36	0·504	9·64	5·2
9	3	0·40	0·560	12·05	4·6
10	3	0·42	0·588	12·15	4·8
				Average =	5·34 %

Thus it is seen that something over 5 per cent. of the nitrogen of lecithin is found in the residue after boiling; this, however, while rather difficult to explain, does not account, to any appreciable extent, for the marked loss in choline-platinum-chloride.

Nitrogen of Filtrate

The determination of the nitrogen in the filtrate was, of course, of great importance; if the filtrate did not contain much more nitrogen than was represented by the choline-platinum-chloride found, it was likely that the loss was due to volatilisation during boiling. The nitrogen of the filtrate plus that of the residue when subtracted from the amount actually present in lecithin represents the amount lost through decomposition and volatilisation during boiling. On an average it was found that the

filtrate contained about 80 per cent. of the total nitrogen of the lecithin used. Thus it will be noticed that the loss due to the formation of volatile products is greater than that in 'lecithol.'

No.	No. of grms. lecithin used	No. of hours boiled	Nitrogen Found: In residue, mgrs.	Nitrogen Found: In filtrate, mgrs.	Nitrogen Found: Total found, mgrs.	Total N of lecithin used	Difference between found and calculated, in mgrs.	Percentage of total N in filtrate
1	0·4393	2	0·58	6·58	7·16	8·12	0·96	81·0
2	0·6721	3	0·76	9·81	10·57	12·43	1·86	79·7
3	0·3114	3	0·54	4·69	5·23	5·76	0·53	81·4
4	0·4219	4	0·49	6·02	6·51	7·8	1·29	77·0
						Average	=	79·78%

Since the filtrate contains about 80 per cent. of the total nitrogen of the lecithin, and since the average amount of choline-platinum-chloride found corresponds only to about 40 per cent. of the theoretical amount, it is obvious that the total amount of nitrogen recovered as the double platinum salt of choline is only about 50 per cent. of the whole. In order to test this directly without the intervention of filtration and other mechanical manipulations by which slight losses might be incurred, the following relative experiments were undertaken. It will be seen that the result is absolutely in accordance with the above, the average percentage found being 50·5 of the total N.

Lecithin was boiled as before for some hours with $Ba(OH)_2$ and the filtrate evaporated to about 60 c.c.; it was then cooled, and excess of acetone added, in order to make sure that no unchanged lecithin was present. A very slight flocculent precipitate formed, which was filtered off, after standing for some time in the ice cupboard. The acetone was then evaporated off and HCl added; $BaCl_2$ was filtered off and the filtrate evaporated to dryness. Residue was dissolved in H_2O, filtered, and filtrate again evaporated to dryness. Residue was now dissolved in absolute alcohol and filtered; it was then re-evaporated to dryness, and re-dissolved in absolute alcohol, and evaporated to 5 c.c. or so; to this solution, which was perfectly clear, about 55 c.c. absolute alcohol was added, and, after thorough shaking, divided by a burette into two parts of 25 c.c. each. One part was run directly into a Kjeldahl flask and the nitrogen estimated; the other was run into a small beaker glass, evaporated to about 2 c.c., precipitated with platinum chloride, and left to stand for twenty-four hours. In this way experiments 1 to 5 in the following table were carried out; experiments 6 and 7 were also done in the same

way only acetone was not used. In experiments 8 and 9 the filtrate was carefully evaporated to dryness without the addition of HCl; the residue was then dissolved in absolute alcohol and to this was added a little HCl; it was then treated as above. This plan was adopted in order to get rid of traces of glycerophosphoric acid which might possibly interfere with precipitation. The results, however, showed, as already found, that this acid does not materially interfere with the separation of the choline as the platinum salt.

	25 c.c. for Nitrogen		25 c.c. for Choline-platinum-chloride	
No.	Nitrogen found, in mgrs.	Choline-platinum-chloride, calculated from N found, in grms.	Amount of Choline-platinum-chloride actually found, in grms.	Percentage of total N found, as platinum salt of Choline
1	5·60	0·1230	0·0619	50·3
2	7·70	0·1692	0·0864	51·1
3	5·88	0·1292	0·0648	50·2
4	8·596	0·1889	0.0983	52·0
5	7·14	0·1569	0·0786	50·1
6	5·04	0·1108	0·0566	51.1
7	5·74	0·1261	0·0624	49·5
8	3·78	0·0830	0·0408	49·2
9	2·80	0·0615	0·0314	51·0
			Average =	50·5 %

Analysis of the above salts gave the following percentages of Pt.:—

Mixture of Nos. 1, 2 and 3	=	31·79 %	Platinum
" " 4 and 5	=	31·93 %	"
" " 6 and 7	=	31·79 %	"

As a result of the above experiments we may assume that, of the nitrogen contained in the filtrate of this lecithin after saponification with $Ba(OH)_2$, not more than about 50 per cent. can be recovered in the form of the double platinum salt of choline. As a control, two experiments with Riedel's 'lecithol' were carried out as above described, when 92·3 per cent. and 91·2 per cent. of the filtrate nitrogen was recovered as choline platinum chloride; these results agree well with those mentioned under 'lecithol,' being, however, a little higher. It is worthy of note that in almost every case the platinum content of the choline salt is slightly above the theoretical amount; possibly it is not quite pure, but contains traces of some other substance. The high content of platinum, however, proves that no part of the lecithin can be present as lecithin-platinum-chloride, otherwise the platinum content should be much lower in other words the lecithin must be split up fairly completely.

Splitting up of Lecithin in Watery Solution

Small quantities of the lecithin were thoroughly ground up in a mortar with barium hydrate, and then boiled for varying periods with enough water to give a saturated barium solution. The flask, which was fitted with a reflux condenser, was at first constantly shaken in order to overcome the marked tendency of the substance to adhere to the sides of the glass above the level of the fluid. The subsequent treatment was similar to that described under lecithol, the choline being obtained as the platinum salt. The following results were obtained by these means; it will be seen that they are practically the same as those obtained with methyl alcohol, the amount of choline-platinum-chloride actually found representing only about 40 per cent. of the total nitrogen of the lecithin used.

No.	Number of hours boiled	Amount of substance, in grms.	Choline-platinum-chloride: Found, in grms.	Choline-platinum-chloride: Calculated from N of lecithin, in grms.	Percentage of theoretical amount
1	2	0·4227	0·0634	0·1718	37·0
2	4	0·5244	0·0856	0·2132	40·1
3	5	0·7678	0·1330	0·3121	42·61
4	7	0·6820	0·1085	0·2773	39·13
5	10	0·4132	0·0676	0·1679	40·26
				Average =	39·82 %

Residue

The residue found after boiling was now examined for nitrogen in the same way as the alcoholic part; here, as before, a certain amount of nitrogen was always found. When compared with the nitrogen of the methyl alcohol residue there was a slight increase, but it was by no means marked; here it may be mentioned that an absolutely exact quantitative determination of nitrogen when dealing with these small quantities is exceedingly difficult, and this may occasionally account for small differences. In making two parallel blind experiments with chemicals alone, it is often noticed that despite the most careful precautions, the results are not *absolutely* the same; in general the difference is so exceedingly slight as not to interfere with the result, but in the above experiments very minute differences interfere to an extent that changes the result to a slight degree.

In these residues the average amount of the total nitrogen found was 8·48 per cent. as shown in the table.

No.	Total nitrogen in lecithin used, in mgrs.	Residue	
		Total nitrogen found, in mgr.	Percentage
1	7·81	0·770	9·86
2	9·70	0·798	8·23
3	6·31	0·448	7·1
4	8·17	0·672	8·22
5	7·62	0·686	9·0
		Average =	8·48 %

Filtrate

Owing to lack of material only one experiment was done in order to test what percentage of the total nitrogen was present in the filtrate: after boiling for three hours the result gave 79·9 per cent. of the total nitrogen: this agrees with that found in the alcoholic filtrate.

Two experiments were made as described on page (54), in which the concentrated filtrates were divided into two exact parts, the nitrogen of one being directly estimated by Kjeldahl's method, and the choline content of the other by platinum chloride. Here, as with methyl alcohol, a little over 50 per cent. of the total nitrogen was recovered as the platinum salt.

No.	25 c.c. for Nitrogen		25 c.c. for Choline-platinum-chloride	
	N found, in mgrs.	Choline-platinum-chloride, calculated from N found, in grms.	Amount of Choline-platinum-chloride actually found, in grms.	Percentage of total N as platinum salt
1	2·59	0·057	0·0295	51·75
2	3·22	0·0707	0·0358	50·6
			Average =	51·18 %

From the above experiments it is seen that in this pure lecithin not more than about 42 per cent. of the total nitrogen can be recovered as the double salt of platinum when the substance is boiled with alcohol or water saturated with barium hydrate: also that not more than about an average of 50 per cent. of the nitrogen in the filtrate can be recovered as choline. A comparison of results obtained by means of similar experiments from 'lecithol' shows that over 77 per cent. of the total nitrogen is recoverable as choline, while a little over 90 per cent. of the filtrate nitrogen is

represented by choline actually obtained as the platinum salt. The following indicates the general relationship as found by a few experiments:—

	Percentage of Total N obtained as Choline-platinum-chloride		Percentage of Filtrate N obtained as Choline-platinum-chloride	
No.	Lecithol	Heart-muscle Lecithin	Lecithol	Heart-muscle Lecithin
1	78·85	41·3	92·3	50·3
2	78·19	42·2	91·2	51·1
3	77·91	39·1	—	52·1
4	76·91	42·0	—	49·5

The result of all these experiments indicates that it is probable that heart muscle lecithin differs in constitution from certain other lecithins with regard to the manner of combination of its nitrogen; that all the nitrogen present is not represented by the choline radical, and that this lecithin contains another nitrogen-containing complex. Investigations bearing on the nature of this nitrogen are at present being carried out.

Cuorin

Cuorin obtained as above described was also examined with regard to its nitrogen-containing radical. This seems much more difficult to hydrolyse than lecithin, but in common with Erlandsen, the writer, after performing several experiments, is of opinion that the base of Cuorin is not choline. The results of certain experiments with this and other substances I hope to give in a later article.

SOME OBSERVATIONS ON THE HAEMOLYSIS OF BLOOD BY HYPOSMOTIC AND HYPEROSMOTIC SOLUTIONS OF SODIUM CHLORIDE

By U. N. BRAHMACHARI, M.A., M.D., *Lecturer on Medicine at the Campbell Medical School and First Physician, Campbell Hospital, Calcutta.*

(Received November 28th, 1908)[1]

In the Lancet for April 2nd, 1904, Sir A. E. Wright and Kilner, in describing a new method of testing the blood and the urine, state that complete haemolysis takes place when a dark coloration is observed in a mixture of one volume of suspension of erythrocytes with one volume of a progressive dilution of a deci-normal sodium chloride solution in a capillary tube. Later on, Wright and Ross[2] point out that instead of making a preparation of the suspension of the red corpuscles, all that is required is to take a measured volume of the blood and to mix with it two volumes of the progressive dilution of the deci-normal sodium chloride solution, and then to observe when the dark coloration takes place.

It will be seen from the above, that it is assumed, firstly, that it is possible to bring about complete haemolysis by mixing one volume of blood with two volumes of a sufficiently dilute solution of sodium chloride, and secondly, that the point of complete haemolysis can be determined by letting light fall obliquely upon capillary tubes containing the mixture, it being supposed to be arrived at when there is a dark coloration of the blood, and there is no bright appearance to be seen in it. In this way, Wright and Ross conclude that the average European blood haemolyses completely with two parts of $\frac{n}{35}$ sodium chloride solution.

I am unable to agree with the observations of Wright, Kilner and Ross, that *complete* haemolysis can be brought about in the above way. By treating normal blood with two volumes of $\frac{n}{100}$ sodium chloride solution as well as with two volumes of distilled water, I have succeeded in demonstrating that in none of these is *complete* haemolysis obtained.

I consider that the most accurate conception of complete haemolysis is that the blood supposed to be completely haemolysed should be perfectly transparent, or if it is not perfectly transparent, it should give, on centrifugalisation, a sediment which, when thoroughly washed with an inactive fluid should not be red. Further, it should not show the presence of

1. Calcutta postmark, November 12th, 1908.
2. *Lancet*, October 21st, 1908.

haemoglobin-containing erythrocytes, which can be stained with proper stains. By an inactive fluid is meant a fluid which has no action on the erythrocytes, and cannot, therefore, dissolve the haemoglobin contained in them, but can dissolve any free haemoglobin.

To determine whether the sediment is *red* or not, the supernatant fluid is to be pipetted off and the sediment treated with a solution of sodium chloride which cannot cause any more haemolysis in the blood under consideration. The mixture is then centrifugalised again and the sediment separated and treated in the same way as before, and if after a sufficient number of washings, it is found that the supernatant fluid at the top is colourless and the sediment is *red*, then it is evident that complete haemolysis has not taken place; the sediment may further be tested for the presence of haemoglobin-containing corpuscles, and stained with a proper stain to show the presence of stained erythrocytes. If, on the other hand, the sediment is colourless, then evidently complete haemolysis has taken place.

Under ordinary circumstances, an $\frac{n}{10}$ sodium chloride solution will serve the purpose of the inactive fluid mentioned above. We shall, however, see, later on, that this solution cannot always be used for the above purpose, as, for instance, when the blood has been previously treated with a saturated sodium chloride solution.

We began our investigations by testing different specimens of blood from the healthy students of the Campbell Medical School, Calcutta. The blood of a large number of students was examined in the above way, the diluting fluid being either distilled water or $\frac{n}{100}$ sodium chloride solution, generally the latter. In none of these cases did I observe complete haemolysis conforming to the definition given above. In other words, I always obtained a *red* sediment after the blood was treated in the above way. At the same time the dark coloration described by Wright and others was generally obtained with two volumes of $\frac{n}{40}$ to $\frac{n}{50}$ sodium chloride solution.

That the red sediment obtained in the above experiments contains undissolved erythrocytes can be shown in the following way:

(1) The sediment though insoluble in $\frac{n}{10}$ sodium chloride solution is dissolved after being repeatedly washed with $\frac{n}{100}$ sodium chloride solution or distilled water as the case may be.

(2) The sediment shows the presence of haemoglobin-containing erythrocytes under the microscope.

(3) The sediment, when stained with a proper stain, shows the presence of stained erythrocytes.

In some of my cases I put the mixture of blood with distilled water, as well as the mixture with $\frac{n}{100}$ sodium chloride solution, for nearly twelve hours in corked tubes, and it was found that complete haemolysis had not taken place even after this period, the temperature of the room being 29°C. during the day.

The question now arises as to how many parts of distilled water or $\frac{n}{100}$ sodium chloride solution can completely haemolyse one part of human blood. I have made dilutions of blood several times with one, two, and up to nine parts of distilled water, as well as $\frac{n}{100}$ sodium chloride solution, and have obtained the *red* sediment in all of them after repeated washing of the sediment with $\frac{n}{10}$ sodium chloride solution. The sediment also showed the presence of haemoglobin-containing erythrocytes, which took easily eosin stain. In one case I diluted a specimen of blood with 40 parts of distilled water, and kept the mixture for twelve hours in a small tube, and could detect the presence of haemoglobin-containing erythrocytes, easily taking the eosin stain.

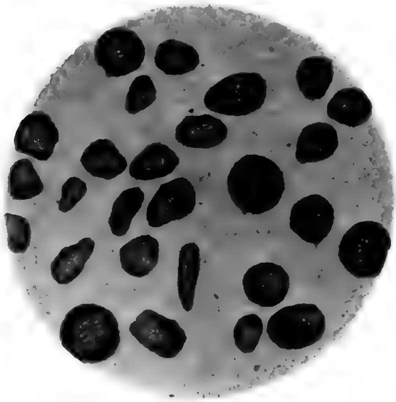
The corpuscles containing haemoglobin, and which are found in the red sediment described above will, in future, be called *sediment corpuscles*.

The sediment corpuscles can be fixed in pure methyl alcohol or absolute alcohol, and stained with a dilute solution of eosin in water, by immersing the slides from twelve to fourteen hours in the solution, or may be stained by mixing the sediment with a dilute solution of eosin in $\frac{n}{10}$ sodium chloride solution.

I append here a plate showing the sediment corpuscles after being fixed and stained in the above manner, they having been obtained by mixing human blood with 2 vols. of $\frac{n}{100}$ sodium chloride solution, and the mixture left undisturbed for one hour at the temperature of the room (29°C.).

A similar phenomenon is seen when blood is treated with nine volumes of $\frac{n}{100}$ sodium chloride solution, except that the sediment corpuscles are much fewer in number and the destructive changes noticed in them are more marked.

The laking of blood by hyposmotic sodium chloride solutions has been supposed to be due partly to osmosis and partly to the specific sensibility of the cortical layer of the erythrocytes or the membranes holding the haemoglobin within the corpuscles. I consider that at least a third factor is present upon which the above phenomenon is to some extent dependent. If we examine the sediment corpuscles, it is easily seen that a large number of them have undergone marked changes in shape, size and in the amount of contained haemoglobin. Some of them are



Sediment corpuscles obtained after treating one part of human blood with two parts of $\frac{n}{100}$ sodium chloride solution. ($\frac{1}{12}$ oil immersion. No. 3 eye piece.) Depth of shading shows amount of haemoglobin. Drawn by B. L. Doss, Calcutta.

resistant in the sense that they have not at all discharged their haemoglobin. But there are others which show marked diminution in the amount of haemoglobin. Some show marked changes in the distribution of their contained haemoglobin, as compared with the normal (see plate). Evidently in these the cortical layer of the envelope has been ruptured, leading to partial escape of the haemoglobin. What is it, therefore, that prevents the remaining portion of the haemoglobin from being completely discharged? The most probable assumption is that the process is to some extent allied to Mass Action that takes place in chemical reactions. In other words, there probably exists a union allied to chemical combination between the haemoglobin of the erythrocytes and other portions of their structure, perhaps including the salts. This combination is probably broken up when water enters their structure, e.g., when they are treated with hyposmotic sodium chloride solutions, but the amount of decomposition will depend upon the relative masses of the interacting compounds.

Resistance of the Erythrocytes to Haemolysis under Abnormal Conditions

If one volume of normal blood is mixed with two volumes $\frac{n}{20}$ sodium chloride solution, slight haemolysis is not infrequently observed, while with $\frac{n}{30}$ it is often distinct or sometimes even marked. In certain forms of anaemia, $\frac{n}{20}$ causes no haemolysis, while $\frac{n}{30}$ causes very slight or no haemolysis. In other words, in some forms of anaemia, the blood resists haemolysis more than normal blood. Captain McCay by estimating the haemosozic value of serum in certain forms of anaemia, also arrives at the same conclusion. He thinks that this might be due to the presence of something of the nature of an antihaemolysin.[1]

Clinical Data of a Case of Anaemia Having High Resisting Power to Haemolysis

Patient (aet. 25) was admitted into my wards on September 16th, 1908. Condition on admission: patient anaemic, slight oedema of the extremities, no albument in the urine, stools contain ova of ankylostomata. On September 22nd, 1908, he had red cells 2,700,000, haemoglobin 20 per cent. One volume of blood plus two volumes of $\frac{n}{20}$ sodium chloride no haemolysis; one volume of blood plus two volumes of $\frac{n}{30}$ sodium chloride—very slight haemolysis. The patient has been treated with thymol since admission, but has been worse since coming into hospital. He is now markedly oedematous, is more anaemic, and his condition is considered hopeless. On November 10th, 1908, his erythrocytes showed more resistance to haemolysis, two volumes of $\frac{n}{30}$ plus one volume of blood not showing the slightest amount of haemolysis. To determine whether this resisting power was due to anything present in the serum, I washed the erythrocytes several times with a deci-normal sodium chloride solution, till the supernatant fluid obtained on centrifugalisation was found to be perfectly free from the slightest trace of of albumen. One volume of the suspension of the erythrocytes was treated with two volumes of $\frac{n}{30}$ sodium chloride solution, the resulting mixture

1. McCay, *Bio-Chemical Journal*, Vol. III, 1908, p. 97.

did not show any haemolysis at all. Salinity was 0·585 per cent., and alkalinity, estimated in the way pointed out by Moore and Wilson,[1] was 0·095 H_2SO_4. Red cells 4,770,000; haemoglobin 13 per cent. It will thus be seen that the resistance to haemolysis was not due to anything present in the plasma, as the same resistance was observed when the serum was replaced by a deci-normal sodium chloride solution. There was a marked diminution in the alkalinity of the blood, which can not, however, account for the resistance of the erythrocytes to haemolysis.

Behaviour of the Erythrocytes of Man and the Rabbit towards Saturated Solution of Sodium Chloride

When one volume of human blood is mixed with fifteen volumes of a saturated solution of sodium chloride in distilled water the mixture at once becomes turbid. This turbidity is quickly followed by a marked solution of the erythrocytes, and the mixture at the same time becomes clear to a great extent. In the rabbit's blood no such clearing up of the mixture takes place in a short time, and the fluid remains turbid for a longer time. If the rabbit's blood, after it has been treated in the above way, be centrifugalised within ten minutes, it is found that the supernatant fluid is faintly red, showing that only a slight haemolysis has taken place by this time, contrary to what is found in the case of human blood in which the supernatant fluid is found to be markedly red. If, however, the sediment of the rabbit's blood obtained above, is mixed with the supernatant fluid at its top, it possesses the remarkable property of dissolving to some extent, showing as it were that some haemoglobin was squeezed out of the erythrocytes during the process of centrifugalisation. The same sediment, when treated with $\frac{n}{10}$ sodium chloride solution, dissolves to a much greater extent. It is thus evident that the undissolved erythrocytes are markedly altered in their constitution after the treatment of the blood with saturated sodium chloride solution. Examined under the microscope they are found to be much contracted, but most of them retain their globular shape and do not look crenated or wrinkled. The most probable explanation of this haemolysis appears to me to be a marked change in the outer walls of the erythrocytes brought about by the sodium chloride of the saturated sodium chloride solution; probably a sort of combination takes place between the sodium chloride and the outer layer of the erythrocytes which finally leads to its destruction. When the blood is mixed with the saturated sodium chloride solution, no

1. Moore and Wilson, *Bio-Chemical Journal*, Vol. I, 1906, p. 297.

doubt water comes out of the erythrocytes by the process of osmosis and they accordingly contract; when the sediment from the above is treated with $\frac{n}{10}$ sodium chloride solution water re-enters their structure, and, as a result of this, they try to expand and regain their original size. But either they burst before or as soon as they recover their original size, or it may be that the water dissolves the compound of sodium chloride and the outer wall of the erythrocytes and consequently a marked haemolysis results. The initial turbidity, mentioned above, is probably due to the production of the compound, which is probably very easily decomposed. It is evident that osmosis alone cannot explain the haemolysis of blood by saturated sodium chloride solution. The remarkable phenomenon of haemoglobin coming out of the corpuscles during centrifugalisation is probably explained by assuming that the damaged walls of the erythrocytes allow haemoglobin to pass out of them by a process allied to filtration under very high pressure. As soon as the unstable compound of the sodium chloride with the outer layer of the erythrocytes is decomposed, the latter behave like small spheres of sponges containing dissolved colouring matter.

FURTHER OBSERVATIONS ON THE ACTION OF MUSCARIN AND PILOCARPIN ON THE HEART

By HUGH MacLEAN, M.D., *Carnegie Fellow, formerly Lecturer on Chemical Physiology, in the University of Aberdeen.*

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(Received December 7th, 1908)

In a former paper[1] I described the parallelism which obtains between vagus inhibition and the effects of muscarin and pilocarpin on the hearts of certain vertebrates. This parallelism has two aspects:—

I—Parallel Distribution of Effects on the Parts of the Heart

All the evidence afforded by my experiments goes to show that in the vertebrate heart (adult) the action of muscarin and pilocarpin reproduces in a remarkable way the effects of stimulation of the inhibitory nervous apparatus, the incidence of the effects on the different portions of the heart being similar in the two cases. Only the parts of any particular type of heart that are supplied with inhibitory nerves are acted on in the characteristic fashion by a suitable dose of muscarin or pilocarpin causing an arrest which, like that induced by vagus stimulation, is set aside by atropin.[2] Thus the ventricle of the eel and tortoise are not acted upon, while that of the frog and newt are, the latter very strongly. It is not simply that the parts endowed with the highest power of spontaneous rhythm, are more readily acted upon by the drugs[3]: the case of the newt's ventricle, with little or no spontaneous rhythmic power, affords important evidence in this connection.

II—Parallel Variation in Effectiveness of Vagus Inhibition and the Two Drugs

Such variation may be due to different causes, such as:—

(1) Seasonal changes associated with the breeding season. Inanition may play some part in frogs and other animals that have been in captivity for considerable periods, but similar changes were observed in recently caught eels.

1. This Journal, Vol. III, p. 1.
2. The drugs were tried both by dropping on the heart and by intravascular injection-methods which, as is known, may not in the case of some drugs yield identical results.
3. See Gaskell's criticism (*Journal of Physiology*, Vol. VIII, p. 408) of Kobert's results (*Arch. f. exp. Pathol. u. Pharmakol.*, Bd. XX, s. 92).

(2) Overdosing with the drugs so that immunity becomes established.[1]

(3) The 'exhaustion' following prolonged stimulation of the inhibitory nerves.

The activity of the inhibitory nervous mechanism was tested by:—

(*a*) Reflex excitation of the vagus.

(*b*) Faradisation of the nerve in the usual way.

(*c*) Faradisation of the sinus in the eel and newt, and of the sino-auricular junction in the frog ('posterior white crescent'); weak and moderate currents were employed, such as when applied in normal heart give the characteristic inhibitory effects readily abolished by atropin.

In the course of my work I was quite aware (in 1905) of the fact that when the usual stimulation of the vagus nerve or the sino-auricular junction in the amphibian heart failed to inhibit whether from absence of inhibitory power depending on seasonal changes, overdosing with muscarin or pilocarpin, exhaustion of the inhibitory apparatus after repeated and prolonged stimulation, &c.—it was still *possible* to arrest the heart beat for very long periods by running up the secondary coil of the induction machine with the electrodes applied in a certain manner to the sino-auricular junction. I did not, however, attach any importance to these results—obtained by the use of such strong currents and differing in various ways from the phenomena of ordinary vagus inhibition—as indications of the condition of the inhibitory nervous apparatus. That faradisation with certain strengths of current can stop the heart in certain conditions when pilocarpin is ineffective, has very lately been noticed independently by McQueen.[2]

Some more recent observations I have made entirely confirm the opinion I then formed in regard to this form of arrest being due to local effects on the cardiac tissue produced by the powerful current and quite different in their nature from true vagus inhibition. The arrest is easily got in the amphibian heart (frog, newt, salamander) when a sufficient strength of current is employed, more especially when the electrodes are made to embrace the sino-auricular junction. Application in the usual way (1-2 mm. apart) to the 'posterior crescent' may also produce the phenomenon, but not with so much facility, as a rule. During the application of the current there is commonly an acceleration of the heart beat, and then, when the current is stopped, a standstill of auricles and

1. See Marshall, *Journal of Physiology*, Vol. XXXI, p. 129.
2. This Journal, Vol. III, p. 402 (Preliminary communication).

ventricle of very variable duration—from a few seconds up to eight or ten minutes or more. Sometimes the period of standstill begins while the current is still applied. The sinus commonly goes on beating regularly during the whole period, but its beats fail to be propagated to the auricles and ventricle, being blocked at the place where the current was applied. Sometimes the sinus stops also. A single stimulus applied to the ventricle during the period of standstill gives a single reversed beat of ventricle and auricles, the contraction failing to pass the blocked area to the sinus. Again, when recommencement takes place, a condition of partial blocking at the faradised area often remains evident for some time, only every second or third beat passing (at first) from sinus to auricle and ventricle. When the current is kept applied for some little time a naked-eye change becomes evident in the faradised area—a distinct whitening or opacity near the electrodes and between them; a feature noted long ago by Wesley Mills as a result of the application of very strong currents. The narrow isthmus of the sino-auricular connection where these blocking effects are readily induced is, of course, of known structural and physiological peculiarity and complexity.

When the heart recommences beating after a period of standstill, the beats are of good strength, as are also beats artificially excited by direct stimulation of the ventricle during the arrest of the normal rhythm. This is, of course, what might be expected in a ventricle which only stopped on account of blocking: there is no sign of the marked depression of contraction force and excitability such as may be induced by vagus inhibition.

When the action recommences there is no increase in force beyond what is shown by the first beat—except such as one may see in the frog-heart as a result of a prolonged period of quiescence (staircase), e.g., resulting from Stannius ligature. Re-application of the current during standstill may excite beats during its continuance: this obviously arises from spreading of current to the auricle. There are various other minor features in the behaviour of the heart under the influence of strong currents, applied in various ways, which hardly seem to call for detailed description—results depending on escape of current to other parts of the organ, electrolytic and thermal effects, &c. Blocking can, of course, be induced in this region, as elsewhere, by other and simpler means which do not involve the same complications.

The same heart may be brought to a standstill repeatedly by applying the current at intervals. There is not the same susceptibility to 'fatigue' that is seen in the case of true inhibitory nerve excitation. Again, a

weak ill-nourished heart seems to be more easily stopped than a vigorous one—in contrast to what has been often noticed in regard to vagus inhibition.

The arrest occurs almost immediately or after some seconds, or, in the case of somewhat less powerful currents, after more prolonged and repeated application. The exact strength of current sufficient to produce these effects varies according to circumstances—conditions affecting current density in the faradised tissue, size and position of electrodes, fulness or emptiness of the organ, the duration of the application of the current, the state of the heart, etc.

With one Daniell cell in the primary circuit and an ordinary Du Bois induction machine, the secondary coil at 7-8 cm. usually suffices; the current is perceptible on the tongue at about 24-25 cm., and causes muscular contraction when applied to a frog's sciatic nerve at 45-50 cm. Such a current is strongly felt on the dorsum of the dry hand. With the small Harvard inductorium the secondary coil has to be moved up to about 4 or 5 cm.; the current is perceptible on the tongue with secondary coil at full distance and inclined to 45°; and stimulates the sciatic nerve with the secondary coil at its full distance and inclined to 85° or 86°. Under certain conditions as to the mode of applying the electrodes, duration and density of current, state of heart, etc., currents weaker than these may produce essentially similar results.

The currents employed to produce the cardiac arrest are quite sufficient, when applied to the frog's sciatic nerve, or skeletal muscle, to cause the depression or abolition of excitability, well known as the Wedensky effect. They are obviously quite unsuitable for testing the condition of the inhibitory nervous mechanism of the heart. It is clear that an incautious strengthening of the faradic current beyond certain limits may lead to confusion between two different forms of cardiac arrest:—

(1) Ordinary inhibition due to excitation of the inhibitory nervous apparatus, and

(2) A stoppage depending on the direct effect of the current on the faradised tissue causing depression, blocking, etc. This phenomenon may be termed *pseudo-inhibition*.

It is by no means impossible that such confusion may have sometimes occurred in the past; it is often difficult in consulting the writings on the subject to be quite sure as to the exact strength of current employed, the duration of its passage, and the precise mode of applying the electrodes; a brief application of current is, *ceteris paribus*, less likely to give the

second-mentioned form of arrest. Burdon-Sanderson, in his 'Practical Exercises,' long ago directed the faradisation to be for a second or less, the points of the electrodes being not more than a couple of millimetres apart, and Porter, in his recent 'Introduction to Physiology' (1906) specifies faradisation 'for a moment.' The effect of atropin on the arrest indicates the type to which it belongs.

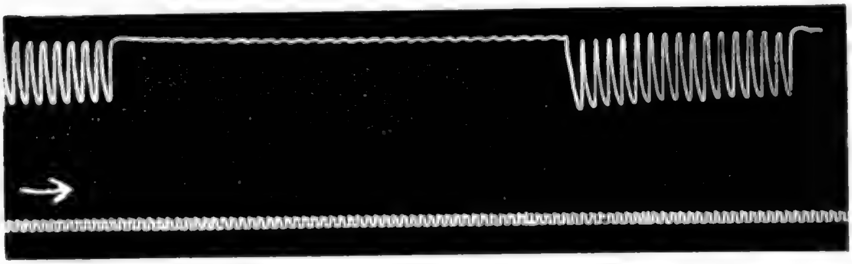

FIG. 1. Faradisation of heart immune to pilocarpin; pseudo inhibition.

Figs. 1 and 2 show tracings of the ventricle of a frog's heart. Fig. 1 is from a heart which was found to be immune to pilocarpin. Faradisation of the white crescent in the usual way with the secondary coil at 10-12 cm. (primary cell 0·9 volt) gave no sign of inhibition. The secondary coil was then moved up to 6·5 cm. and standstill resulted, as seen in the tracing—beginning when the current was stopped after being applied for about fifteen seconds. Fig. 2 shows a similar standstill in the same heart after a large dose of atropin. The sinus action continues in each case, though much more visible in the tracing in Fig. 1. The time tracing shows seconds in each case.

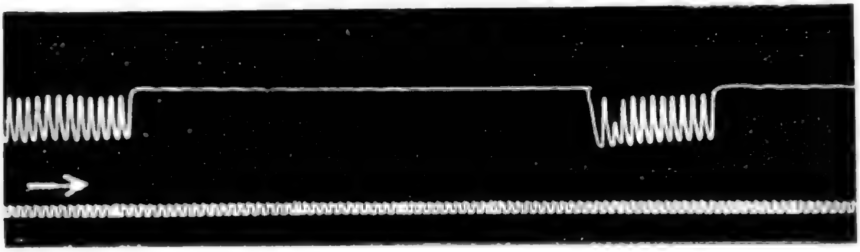

FIG. 2. Faradisation of the same heart after atropine; pseudo-inhibition.

The parallelism between the effects of muscarin and pilocarpin and those of electrical stimulation of the inhibitory nerves is not affected by the results described above obviously due as the latter are to the

depressing effects of the excessively strong current on the cardiac tissue (causing blocking, etc.) and not affected by atropin—in contrast with what holds good in regard to the inhibitory influence excited by faradisation of the sinus in the eel and newt or of the sino-auricular junction in the frog when done in the ordinary way with moderate currents, an experiment long familiar to physiologists.

It is known that atropin also abolishes the local inhibitory effects produced by weak faradic currents in the auricle of the tortoise and eel, and in the auricle and ventricle of the newt. Evidence has been adduced by Gaskell[1] in the case of the tortoise's auricle, and by MacWilliam[2] in that of the eel, showing that these local inhibitory effects are due to excitation of inhibitory fibres in the auricular wall and not to direct effects on the muscle. The exact place of attack by atropin (whether on 'nerve endings' or muscle proper) is immaterial in the present connection; the essential point is that the influence of inhibitory fibres (preganglionic or postganglionic) is cut out by atropin. Even strong currents are then unable to cause the usual inhibitory arrest in a state of relaxation which is, in ordinary circumstances, easily and strikingly obtained in the eel's auricle and newt's ventricle.

In the light of the evidence available from various sources regarding the action of muscarin and pilocarpin, it is clear that their effects are closely bound up with the functional efficiency of the inhibitory nerves, and their special distribution in the various parts of the vertebrate heart. The effects of the drugs are, with most probability, to be ascribed to a 'stimulating' influence (by chemical interaction, no doubt) on some part of what, for the sake of brevity, has been termed 'nerve endings' of the vagus (postganglionic) fibres, i.e., on some part of the linkage between the nerve fibre and the fundamental contractile mechanism of the muscular fibre (myoneural junction, etc.).

I have to thank Professor MacWilliam for the facilities he has kindly afforded me for carrying out my experiments in his laboratory.

1. Schäfer's *Text-book of Physiology*, Vol. II, p. 208.
2. *Journal of Physiology*, Vol. VI, p. 228.

THE OCCURRENCE AND DISTRIBUTION OF CHOLESTEROL AND ALLIED BODIES IN THE ANIMAL KINGDOM

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A large number of observations, accumulated in the course of the past fifty years, have shown that cholesterol is a constant constituent, so far as they have been examined, of all animal tissues. The investigations recorded relate chiefly to man and a few of the more common mammals and birds, in all of the organs of which, and in most of their fluids and secretions, cholesterol has been found. It is evident that the constant presence of such a substance indicates its great importance from a vital standpoint, and necessitates its recognition as a primary constituent of all protoplasm. This conclusion has recently been fully emphasised by various writers, who have called into question the dictum of Pflüger 'Nur das Eiweiss ist lebendig,' and have assigned to the lipoid class of bodies, of which cholesterol is one of the most important members, functions second only in importance to those of the proteins themselves. What these functions may be in the case of cholesterol we do not know. They would, one must suppose, be quite different from those of the proteins, for whereas these bodies are, from a chemical point of view, unstable, being capable of rapid transformation into innumerable other substances, more or less complex as required by the life of the organism, cholesterol is characterised chemically by a remarkable stability. That it is constantly associated in the cell with lecithin has long been known; that lecithin can assist the action of various poisons which act by producing haemolysis, and that cholesterol, on the other hand, functions as an antitoxin in this respect, are facts which have recently come to light, and in them, possibly, may be found a clue to the important question of the part played by cholesterol in the life of the organism.

It is a remarkable fact that while cholesterol has been isolated uniformly from the tissues of mammals and birds, whenever the lipoid bodies contained in them have been investigated, no other substance similar to it had, until quite recently, been discovered in animal protoplasm.[1] From the vegetable kingdom, on the other hand, a large

1. With the exception of the isocholesterol of Schülze, which will be discussed in the sequel.

number of bodies, isomeric with, and no doubt closely related to, cholesterol, have been obtained the phytosterols. Although recent observation shows that the number of these is not so great as previously supposed, owing to the fact that many of those described under different names are mixtures of phytosterol itself, which occurs in a pure form in wheat germ,[1] with other allied bodies, still, not only do many different vegetable cholesterols exist, but, frequently, different forms occur in the same plant, each associated with one or other of the plant structures. With cholesterol these various phytosterols make up the cholesterol group. Except in the case of cholesterol itself, we have little or no knowledge of the chemical nature of any of these bodies, but, for the present purpose we may consider the cholesterol group as consisting of:—

(*a*) A number of isomeric secondary alcohols $C_{27}H_{46}O$, which are all unsaturated, and possess one well-defined double link in the molecule. These are all laevorotatory and give the colour tests of Salkowski and Liebermann.

(*b*) A number of bodies, almost entirely of vegetable origin, which are undoubtedly similar to those of the preceding class, but which differ from them in their general properties, and, probably, are not isomeric with cholesterol. Their relationships to cholesterol and to one another are entirely unknown.

(*c*) The natural derived product coprosterol, with which, for reasons given below, may be classed isocholesterol.

If, then, cholesterol is a body which is one of the primary constituents of animal protoplasm, we should expect to find it not only in the highly organised animals, but throughout the series from Chordata to Protozoa or if cholesterol itself were not present its place should be filled by other and closely related forms. In the latter case it might be found that each of the great classes of the animal kingdom was characterised by the presence of a different member of the cholesterol group. On the other hand, if cholesterol is not of primary importance to all forms of life, it is not impossible that animals might be found into the composition of whose protoplasm it did not enter. In regard to the lipoids contained in the lower animals our information is very scanty. In the older literature we find occasional references to cholesterol as a constituent of one or other of these, but no precise identification of the body was, as a rule, made. Quite recently the work of Henze[2] on the Sponge, *Suberites domuncula*,

1. Burian, *Monatshefte*, XVIII, 553.
2. *Zeit. physiol. Chem.*, 1908, LV, 427.

and that of Menozzi and Moreschi[1] on the pupae of *Bombyx mori*, have shown that in the invertebrata there exist cholesterols which differ from the common cholesterol of the vertebrata. The present writer, on the other hand, in a preliminary investigation,[2] found that in two species of sea anemone (Coelenterata), cholesterol itself was present, and with a view to throwing more light on the distribution of cholesterol in the animal series, and to solve, if possible, some of the questions referred to above, it was decided to examine the cholesterol bodies contained in a number of animals typical, so far as possible, of each of the great sub-kingdoms of Animalia. An account of these experiments will be given in the following pages.

Classification and Types Selected

The types selected for experiment may be classified as follows:—[3]

CHORDATA—

Mammalia	*Lepus cuniculus*, the rabbit.
Reptilia	*Tropidonotus natrix*, the grass snake.
Pisces	*Scomber scombrus*, the mackerel.

MOLLUSCA

Gastropoda	*Buccinum undatum*, the whelk.

ARTHROPODA—

Crustacea	*Carcinus mænas*, the crab.
Insecta	*Blatta orientalis*, the cockroach.

ANNULATA—

Chœtopoda	*Lumbricus terrestris*, the earth worm.

ECHINODERMATA

Asteroidea	*Asterias rubens*, the starfish.

COELENTERATA—

Actinozoa	*Tealia crassicornis*, *Actinia equina*, } sea anemones.

PORIFERA

Cliona celata.
Ephydatia fluviatilis, *Spongilla lacustris*, } fresh water sponges.

1. *Atti della R. Accad: dei Lincei* [V] XVII, 95.
2. *Proc. physiol. Soc.*, XXXVII, July, 1908.
3. Based upon the system given by Parker and Haswell, *Text Book of Zoology*, Vol. I, Macmillan, 1897.

Method of Experiment

The animals were, if necessary, killed with chloroform, and then ground up in a mortar with coarse sand, plaster of Paris being added from time to time to dry up the crushed material. If the tissues were very tough, as in the case, for instance, of the starfish, they were first passed through a mincing machine, all juices expressed being collected in plaster of Paris. The mass so obtained was left till it had become perfectly dry and hard, after which it was coarsely powdered, and then extracted in a large Soxhlet apparatus with ether, for periods which varied between seven and fifteen days. The ether solution so obtained was at once saponified with a large excess of alcoholic solution of sodium ethylate, according to the method of Kossel and Obermüller. To ensure complete saponification the liquid was allowed to stand at least twelve hours, after which the precipitated soaps were filtered off and well washed with ether. The filtrate was then shaken several times with water to remove traces of soap, the ethereal solution dried over calcium chloride and the ether distilled off.

The crude unsaponifiable residue so obtained is generally described as cholesterol, and weighed as such. But in the case of animal extracts, it always contains brownish oily, or resinous substances, which may constitute as much as three-quarters of the total residue. For the purpose of the present work it was necessary to isolate the cholesterol in a pure state so that its identity could be established beyond question. If the quantity of residue was sufficient it was dissolved in absolute alcohol, filtered, the hot alcohol diluted to about 90 per cent. strength and the liquid allowed to crystallise. A microscopic examination at this point gave valuable indications of the presence of cholesterol, pure or in an admixed state. After the separation of crops of crystalline matter, the filtrates, evaporated to dryness (or if very small the original unsaponifiable material), were treated by the following method, in which the cholesterol is isolated in the form of cholesterol benzoate. The dried residue was dissolved in pyridine (20 c.c. to each gram of substance), and an excess of benzoyl chloride dissolved in pyridine added and the mixture allowed to stand overnight. In this way the cholesterol is converted quantitatively to benzoate.[1] The pyridine solution was then poured into water, which precipitated the organic matter, and the whole allowed to stand, if necessary, until the colloidal solution, which at first formed, coagulated. This usually happened in a few hours, but very occasionally

1. Dorée and Gardner, *Proc. Roy. Soc.*, Series B, 1908, LXXX, 228.

salting out was necessary. After washing and drying the precipitate it was boiled out with absolute alcohol, the liquid allowed to cool, and the benzoate filtered off. Cholesterol benzoate is very insoluble in alcohol in the cold according to a determination made for the author, 100 c.c. of commercial absolute alcohol dissolve only 0·12 gram at 20° C.—so that the unchanged oily matters can be completely separated from the benzoate, and if the quantity of alcohol used is measured, a correction can be applied to the quantity of benzoate obtained. The benzoate crystallises well from ethyl acetate in large rectangular plates.

The formation of a benzoate by this method serves in itself for the recognition of cholesterol, since, so far as the author's investigations at present carry, vegetable cholesterols do not benzoylate, or if so, imperfectly under the conditions described. But the characteristic properties of cholesterol benzoate enable it to be identified with certainty. It dissolves with difficulty in alcohol, from which it crystallises in *square* plates; it melts at 145° C. to a *turbid* liquid, which clears suddenly at 178° C., and this on cooling, shows a brilliant display of colours, of which a light blue at the higher temperature followed by a deep violet at the lower are very characteristic. Some other benzoates of members of this group are now known to give colours on solidifying. One being the sitosterol of Burian,[1] the colour phenomena of which are described by Ritter,[2] and the others the new sponge cholesterols described in the present paper. The colour phenomena of these, however, are quite different from those shown by cholesterol benzoate.[3]

A second important method for the isolation and identification of cholesterol consists in its conversion to a dibromide by the method of Windaus.[4] In this process one gram of the substance dissolved in 10 c.c. of ether is mixed with a solution of 0·5 gram of bromine dissolved in 5 c.c. of glacial acetic acid. The mixture is allowed to stand at 0° C., when a crystalline precipitate of cholesterol dibromide forms, which is filtered off and washed successively with acetic acid, 50 per cent. acetic acid and water, after which it is pure. By this means an almost complete separation of cholesterol from, at any rate, vegetable cholesterols is possible. Whereas 100 c.c. of the ether glacial acetic acid mixture dissolve only 0·6 gram of the dibromide at 20° C., phytosterol dibromide

1. *Loc. cit.*; from wheat germ. This body should now be called phytosterol.

2. *Zeit. physiol. Chem.*, XXXIV, 431.

3. The colours may be observed very clearly if the benzoate is melted in a thin layer between two glass plates. As the cooling is comparatively slow the order of the colours can be exactly noted.

4. *Ber.*, 1906, XXXIX, 518; *Chemiker Zeit.*, 1906, XXX, 1011.

is very soluble. This reaction, until recently, was characteristic for cholesterol, but it has lately been found that the bombicesterol of Menozzi and Moreschi, and the sponge cholesterols described below also give the reaction in the same way as cholesterol. Since all these dibromides give the same analytical figures, the melting points become important as a means of identification. Windaus gave the melting point of cholesterol dibromide, prepared and purified by his method as 123° C. Bondzyński and Humnicki,[1] who prepared it by the addition of a solution of bromine in light petroleum to a solution of cholesterol in the same solvent, found 109° C. as the melting point. Menozzi and Moreschi again,[2] in an examination of the cholesterol from hen's eggs (which proved identical in all respects with the cholesterol from gall stones), found that the dibromide prepared according to Windaus' instructions, but subsequently crystallised from alcohol, melted at 111° C. The figure obtained for the bromine content of this body agreed closely with that required for cholesterol dibromide, so that the substance was not altered in constitution by treatment with alcohol. The present writer, on the other hand, has found difficulty in obtaining it from alcohol in a crystalline form, but in order to decide the question of the melting point a sample of cholesterol was prepared from human gall stones in the usual way and converted to the dibromide by Windaus' method, the instructions as to washing, etc., being exactly followed. The pure white substance was, without further purification, dried in vacuo, and found to melt at 123° C., decomposing at a few degrees higher. Windaus' statement is thus perfectly correct. Clionasterol dibromide, prepared and washed in an exactly similar way, melted sharply at 114° C., and decomposed rapidly between 116° and 120° C. The melting point of bombicesterol dibromide, similarly made, is stated to be 111° C.

Systematic Examination of Types Selected

In the protoplasm of vertebrate animals cholesterol is universally present. In man it is especially abundant in the brain (2·5 per cent.) and nervous tissue (1 per cent.), while in the fat it is found to the extent of 0·35 per cent. and in dry muscle 0·23 per cent. It is also present in bile, 0·07 per cent., in blood, 0·09 per cent., and in milk, 0·032 per cent. In herbivorous animals, whose food contains no cholesterol, the figures, so far as they are available, follow very much in the same order. The

1. *Zeit. physiol. Chem.*, XXII, 396.
2. *Atti della R. Accad. dei Lincei* [V] XVII, 91.

fact that the cholesterol obtained from various animal sources is one and the same substance, has been demonstrated by Menozzi,[1] who made a careful comparison of the physical and chemical properties of the cholesterol isolated from cow's milk, from horse brain, and from hen's eggs. Recently, also, Diels and Linn,[2] on account of an apparent slight variation in the chemical behaviour of cholesterol obtained from egg-yelk, made a comparison of this specimen with others obtained from gall stones and from brain, respectively, and showed that when properly purified the melting point and rotation of the cholesterol from each of these sources was substantially the same.

Cholesterol is the only body of its kind so far found in connection with the higher vertebrate animals, with the exception of the so-called iso-cholesterol, to which reference has already been made. This has only been obtained from the wool-fat of the sheep.[3] It differs markedly from cholesterol in its properties, and seeing that it is a product excreted by the skin, may, perhaps, be classed with coprosterol, which is a derivative of cholesterol, normally excreted in the faeces by men,[4] and by carnivorous animals when fed on a raw brain diet.[5] The relation of coprosterol to cholesterol is at present unknown, but coprosterol, unlike cholesterol, is a saturated compound, and probably contains two hydrogen atoms more in the molecule. It is not, however a simple reduction product of cholesterol, and, most probably, has a somewhat different carbon skeleton. But the physical properties of coprosterol stand in marked agreement with those of iso-cholesterol. Alone among the known members of the cholesterol group, iso-cholesterol does not give the characteristic colour-reaction with sulphuric acid and chloroform (Salkowski's test): coprosterol gives it in a modified way. Both these bodies too, are, unlike all the others, dextro-rotatory. An alteration in rotatory power from negative to positive has been observed generally to be brought about by the saturation or modification of the side chain of cholesterol, which contains the double bond. The dextro-rotatory power of coprosterol is, no doubt, due to such a modification, but whether that of iso-cholesterol can be ascribed to a similar change is less probable, since, according to Darmstädter and Lifschütz,[6] it is, like cholesterol, unsaturated, readily absorbing bromine in chloroform solution. Iso-

1. *Atti della R. Accad. dei Lincei* (V) XVII, 91.
2. *Ber.* 1908, XLI, 260.
3. Schülze, *Ber.* V, 1075; VI, 251; XII, 249.
4. *Zeit. physiol. Chem.*, XXII, 396.
5. *Proc. Roy. Soc.*, B. LXXX, 228.
6. *Ber.*, 1898, XXXI, 97, 1122.

cholesterol is still further distinguished from cholesterol and its isomers by the melting point and crystalline form of its acetate and benzoate. While the benzoates of the latter all melt at about 145° C. and crystallise from alcohol in rectangular plates, the benzoate of iso-cholesterol melts at 191° C. and crystallises from alcohol in needles. These facts are collected together in the following table:—

	Crystal form (dilute alcohol)	M.p.	$[a]_D$ (ether)	Acetate M.p.	Acetate Crystal form	Benzoate M.p.	Benzoate Crystal form
Cholesterol	Oblong plates	147°	−31°	114°	Plates	145°	Plates
Isocholesterol ...	Flocks	137°	+60°	Below 100°	Amorphous	191°	Needles
Coprosterol	Needles	100°	+24°	88°	Needles	122°	Plates

The presence of cholesterol in the blood and eggs of birds has been frequently observed. In the case of reptilia its occurrence has not, apparently been recorded, but it is well known as a constituent of oils obtained from various species of fish. Dog-fish oil, for example, is said to contain four to five per cent. of cholesterol. In order to investigate this point, and to ascertain whether any other member of the cholesterol group was present in the tissues of reptiles and fishes, an experiment was carried out with a typical representative of each of these classes. For purposes of comparison the cholesterol contained in the whole body of a small mammal (rabbit) was estimated by a similar process.

CHORDATA. Mammalia. *Lepus cuniculus*, the rabbit.—A rabbit weighing 2·8 kilos. was killed, and the blood, which weighed 75 grams, was collected separately, mixed with sand and plaster of Paris, and, when dry, ground to powder; the rest of the animal, including the fur, was then passed several times through a sausage machine, the minced material being ground up with coarse sand, and dried with plaster of Paris. The total mass obtained was extracted for twenty days with ether, and the solution saponified with sodium ethylate and washed as already described. The total unsaponifiable matter weighed 6·0 grams. It was at once dissolved in pyridine and treated with an excess of benzoyl chloride. The product which was obtained on precipitation with water was boiled out with absolute alcohol, in which it was very difficultly soluble, and after re-crystallisation from ethyl acetate appeared in the typical crystalline form of cholesterol benzoate. In all 4·112 grams of benzoate were obtained, which melted correctly and showed the characteristic colour play. This corresponds to 3·239 grams of cholesterol, or 0·117 per cent.

A microscopic examination of the residues soluble in alcohol

(consisting of brown oily matter which slowly became resinous) revealed no signs of any other crystalline matter.

REPTILIA. *Tropidonotus natrix*, the grass snake. Four grass snakes, weighing 246 grams, were killed, passed through the mincing machine and ground up with sand and plaster of Paris. The dry mass was extracted for eight days with ether, and the pale yellow extract saponified with sodium ethylate. A large quantity of a pale brown soap separated, which was filtered off and washed. From the filtrate, after the usual treatment, 0·44 gram of unsaponifiable matter was obtained, which was usually free from colour. It was dissolved in alcohol and crops of crystals weighing 0·12 gram were separated. These, after re-crystallisation, melted sharply at 145° to 146° C., and under the microscope showed the characteristic crystal form of cholesterol. On benzoylation of the residues in pyridine solution 0·09 gram of benzoate was isolated. This was very difficultly soluble in alcohol, from which it crystallised in square plates. It melted at 144° to 145° C. to a turbid liquid, which cleared at 178° C., and on cooling showed the colour play of cholesterol benzoate. The total yield of cholesterol was thus 0·21 gram, or 0·08 per cent. No other similar body was observed in the residues.

PISCES. *Scomber scombrus*, the mackerel.—Five mackerel, weighing 1,452 grams, were ground up in a mortar with coarse sand mixed with plaster of Paris, and allowed to dry. The mass was then reduced to a coarse powder and extracted in a Soxhlet apparatus for eight days. After washing in the manner described above, the ether solution was pale yellow, and on evaporation left 1·07 grams of brownish crystalline matter. This was dissolved in 90 per cent. alcohol, and under the microscope showed perfectly formed, typical, cholesterol crystals, no sign of any other crystal or mixed form being observed. A small crop, weighing 0·08 gram, was isolated, re-crystallized from acetone, and found to melt at 145° to 146° C. The whole filtrate from this was benzoylated in pyridine solution and yielded 0·37 gram of a benzoate, which, after re-crystallisation from acetic ether, appeared in the form of shining rectangular plates. These melted at 145° C. to a turbid liquid, which became clear at 180° C., and showed the colours characteristic of cholesterol benzoate. The alcoholic filtrate from the crude benzoate weighed 0·43 gram, and consisted of a brown resin which, on long standing, only showed minute traces of crystalline matter. The cholesterol obtained was thus about 0·42 gram, or 0·03 per cent., and no other similar body was observed.

MOLLUSCA. GASTROPODA. *Buccinum undatum*, the whelk. A number of whelks, weighing 1,179 grams after removal of the shells, were minced up and treated with sand and plaster of Paris, the mass extracted for six days with ether, and the extract saponified as described above. The soaps were considerable in quantity and dark brown in colour; the filtrate was also dark brown, and on evaporation left 4·5 grams of beautifully crystalline unsaponifiable matter (0·38 per cent.). This was dissolved in dilute alcohol, and a microscopic examination showed the presence of typical cholesterol crystals, the same crystalline forms being observed down to complete dryness. Three crops of white crystals, weighing 0·75, 0·25 and 0·09 gram were separated and found to melt at 142° to 143° C. The whole 1·09 grams put together and re-crystallised from acetone, melted at 144° to 145° C., and appeared as pure cholesterol. The whole of the residues from these were dissolved in pyridine and treated with benzoyl chloride in the usual way. By this means 0·37 gram of cholesterol benzoate was obtained, which, after re-crystallisation from ethyl acetate, melted at 145° C. to a turbid liquid, which became clear at 185° C., and on cooling showed the characteristic colour play. A sample of the pure cholesterol dissolved in ether was then treated with a solution of bromine in glacial acetic acid. In a few minutes the solution set almost solid. The precipitated dibromide, after washing and drying in the usual way, melted at 118° to 119° C. with decomposition, in which respect it agreed fairly well with cholesterol dibromide. The total yield of cholesterol was thus approximately 1·46 grams, or 0·124 per cent.

The residue left after separation of the benzoate was a clear, brown oil, which, after standing for six months, contained no trace of crystalline matter, and showed no signs of solidification.

CEPHALOPODA. Henze[1] has recently shown that the hepato-pancreas of *Octopus vulgaris* contains fats and 'not inconsiderable quantities' of cholesterol.

ARTHROPODA. CRUSTACEA. *Carcinus mænas*, the edible crab.—A whole crab, weighing 538 grams, was ground up with sand, etc., and extracted for eight days with ether. On saponification of the extract a considerable quantity of a stiff reddish coloured soap was obtained. The filtrate from this, after washing, was pale yellow, and left 1·0 gram, or 1·9 per cent., of crude residue. This, dissolved in 90 per cent. alcohol, left a small quantity of brown insoluble substance. A microscopic

1. *Zeit. physiol. Chem.*, 1908, LV, 438.

examination of the solution showed abundance of typical cholesterol crystals, and from it 0·25 gram of nearly pure cholesterol was ultimately separated. After re-crystallisation from acetone, it was pure white, and melted sharply at 146° to 147° C. Its identity with cholesterol was confirmed by the preparation of the following derivatives:

(*a*) *The dibromide*: 0·12 gram of substance was dissolved in 2 c.c. of ether and mixed with 0·6 c.c. of Windaus' solution of bromine in glacial acetic acid. The mixture set almost immediately to a mass of crystals, which were filtered off and washed. After drying in vacuo, these melted at 120° C., turning brown and decomposing at 124° to 126° C. The filtrate on treatment with water deposited a white solid, which contained bromine, and melted at 100° C., decomposing at 120° C.

(*b*) *The benzoate*: This was prepared by heating the dried substance with benzoyl chloride to 165° C. for five minutes. The residue was boiled out with alcohol, and the insoluble white crystalline residue purified by crystallisation from ethyl acetate, after which it melted at 145° C. to a turbid liquid, became clear at 178° C., and on cooling showed the colour play characteristic of cholesterol benzoate.

The cholesterol isolated was, therefore, about 0·25 gram, or 0·048 per cent.

INSECTA. *Bombyx mori*, the silkworm moth.—Owing to the fact that an oil has been obtained commercially from the *pupae* of this moth, several investigations have been made with a view to decide whether the unsaponifiable residue of the 'chrysalis oil' contains cholesterol—as do other animal oils—or a phytosterol. Lewkowitsch,[1] who first examined the question, came to the conclusion that cholesterol was present in the oil, since, by the use of Bömer's acetate method, he was able to isolate from it an acetate whose melting point, after successive crystallisations, finally stood at 114° C. M.Tsujimoto,[2] however, who has recently made a further investigation, obtained 1·63 per cent. of unsaponifiable residue from the oil, from which, after repeated purification, he prepared a substance of M.p. 143° C., which in crystalline form and the melting point of the acetate (125° C.) corresponded rather with phytosterol.

Realising that the question of the presence or absence of cholesterol could be definitely decided by the application of the methods described in the introduction to this paper, a quantity of the pupae was obtained from France with a view to the extraction of the cholesterol body from

1. *Zeit. fur Nahr. u. Genussmittel*, 1907, XIII, 552.
2. *Journ. of the Coll. of Engineering*, Tokyo, Japan, 1908, IV, 63.

them on a large scale, when a paper by Menozzi and Moreschi[1] appeared which rendered the proposed investigation unnecessary. These authors showed that chrysalis oil contains 10 per cent. of its weight of unsaponifiable matter, and that in this there are probably at least four different substances, two of which are paraffin hydrocarbons. One of these hydrocarbons has apparently the formula $C_{28}H_{58}$, and a melting point of 62°. The main constituent, however, is a new isomer of cholesterol, to which the name of bombicesterol has been given, and which, in all its properties, bears an extraordinarily close resemblance to cholesterol itself. The melting point and rotation of the two bodies, and of their benzoates correspond exactly with one another, and the melting points of the dibromides (111° C.) are stated to be the same. The crystalline form of bombicesterol differs somewhat from that of cholesterol, but the most important difference between the two lies in the melting points of the formiates and acetates. Bombicesterol formiate melts at 101° C. (as compared with 96° C.), and the acetate at 129° C. (as compared with 114° C.). The benzoate is said to show on melting the phenomenon of liquid crystals, but apparently this is not accompanied by a play of colours, as in the case of cholesterol benzoate.

The authors further mention that from the crude acetate of bombicesterol they obtained, by the method of Windaus and Hauth, two dibromides. One of these remained in the ether acetic acid solution, and was thrown out on the addition of water. On reduction it yielded bombicesterol acetate, melting at 129° C. The other dibromide crystallised out at once in the ether acetic acid solution, and on reduction gave an acetate of melting point 114° C., which is the same as that of cholesterol acetate. The point is apparently still under investigation, but it is obvious that the possibility of the presence of cholesterol in the pupae of *Bombyx mori* is not wholly excluded.

In order to discover whether the *larvae* of *Bombyx mori* contained cholesterol, a quantity of the worms, weighing 102 grams, were kept without food for three hours and then killed. On grinding them up with sand a strong leafy smell was observed. The dried mass was extracted for five days with ether, and the deep green extract saponified. The soap was moss green in colour, this being, no doubt, due to the presence of chlorophyll. The green filtrate from the soap, also, on washing, became pale yellow, all the green colour going into solution in water. The unsaponifiable residue weighed 0·1 gram (or 0·1 per cent). With this

1. *Atti della R. Accad. dei Lincei*, [V] XVII, 95.

small quantity little more than a microscopic examination was possible. The residue was dissolved in 90 per cent. alcohol, and a drop of the solution was allowed to crystallise on a slide. At first, long, very narrow plates, not quite rectangular, were seen, which, although quite different from those of cholesterol, answered to the description of the crystalline form of bombicesterol (*lamine allungate ed acuminate*) given by Menozzi and Moreschi. The later crystals were in the form of long, narrow hexagons, agreeing in this respect with those of the vegetable cholesterols. They probably consisted of the phytosterol of the mulberry leaves on which the worms were fed.

The residue was crystallised from methyl alcohol,[1] in which it was very insoluble, and the small crops of white crystalline matter obtained melted at 125° C., but not sharply.

The *eggs* of *Bombyx mori* were examined in 1885 by Tichomiroff.[2] The eggs, which are laid in the summer, develop up to a certain point, pass the winter in this state, and continue development in the spring. Tichomiroff, using Hoppe-Seyler's methods, obtained the following percentage figures: (A) for eggs which had reached the winter stage; (B) for eggs on the point of hatching:—

	Fat	Lecithin	Cholesterol
A	8·08	1·04	0·40
B	4·421	1·76	0·35

As, however, no examination was made of the 'cholesterol' obtained, its identity with either cholesterol or bombicesterol must remain uncertain.

Blatta orientalis, the cockroach.—Cockroaches, weighing 194 grams, were killed and dried in the steam oven, after which the weight was 65·5 grams. The solid matter was ground to powder with a little sand, and extracted with ether for fourteen days. The brownish ether extract on saponification gave a brownish coloured soap in considerable amount, which was very solid when dry. The unsaponifiable residue, which was very liquid at 100° C., weighed 0·5 gram. It was treated with absolute alcohol in which a part readily dissolved, leaving an insoluble portion, which, on warming the liquid to about 40° C., melted, forming heavy oil drops. By decanting off the alcohol from these and repeating the process several times, a separation of the insoluble and the soluble parts was effected.

A. The part insoluble in alcohol weighed about 0·2 gram. It was

1. The methyl alcohol used here and elsewhere was purified, but not absolute.
2. 'Chemische Studien über die Entwicklung der Insecteneier.' *Zeit. physiol. Chem.*, IX. 525.

moderately soluble in petroleum ether, and easily so in benzene. From the former solvent it came out as a soft, white, buttery solid, and was not apparently crystalline. On heating slowly in a wide capillary tube, it melted at 42·5° to 43° C., and solidified sharply at 40·5° C. When warmed with pyridine it dissolved, but on cooling, the solution set to an opaque gelatine-like mass. It did not give Salkowski's test and did not absorb bromine. This substance, therefore, is very probably a hydrocarbon similar to those found in the pupae of the silkworm moth.

B. The part easily soluble in alcohol. The alcohol was diluted to about 90 per cent. strength, and on allowing a drop of the solution to crystallise under the microscope, thin plates closely resembling those of cholesterol were observed. As the substance was somewhat soluble, even in the diluted alcohol, methyl alcohol was used as a solvent, and from this after repeated crystallisation, about 0·1 gram of crystals, with a constant melting point of 139° to 140° C. was obtained. That these were not cholesterol was shown by the following tests:—(i) When moistened with concentrated sulphuric acid the edges of the crystals did not turn red. (ii) On benzoylation in pyridine solution no action took place, as the substance easily soluble in alcohol, crystallising in oblong plates and melting sharply at 139° to 140° C., was recovered unaltered.

The body gave Salkowski's test with chloroform and sulphuric acid exactly as cholesterol, but owing to the small quantity of the pure substance that could be isolated, it was not possible to characterise it further. It would, however, appear to be similar in its general behaviour to other members of the cholesterol group.

ANNULATA. CHŒTOPODA. *Lumbricus terrestris*, the earth-worm. —Earth-worms,[1] weighing 286 grams, were killed and ground up with sand and plaster of Paris. The dry mass was extracted for five days with ether, and the extract, on saponification, gave a considerable quantity of a pale brown, slimy soap. The filtrate from this yielded 0·95 gram of unsaponifiable residue, which was practically solid at 100° C. It was soluble in 90 per cent. alcohol, with the exception of a trace of brownish matter. A microscopic examination showed very badly-formed cholesterol plates, together with a few long, blunt-pointed, needle-shaped crystals. Crops weighing 0·1 gram and 0·22 gram, respectively, were separated, and, after re-crystallisation from acetic ester, melted at 142° to 143° C., and consisted largely of plates. The 0·31 gram of substance thus obtained was benzoylated in pyridine solution in the usual manner.

1. The worms used were the lob or dew worms of the freshwater fisherman.

The benzoate obtained was very insoluble in alcohol, and after re-crystallisation from acetic ester, melted at 144° to 145° C. to a turbid liquid, which became clear at 180° C., and on cooling showed a brilliant colour play. The filtrate from this was examined under the microscope, when, beside a few crystals of the benzoate, a number of minute spherular crystals were observed.

All the residues, consisting of a dark brown, sticky resin, were put together, dried, and treated with benzoyl chloride in pyridine solution. No crystalline matter, however, could be obtained from them. The total yield of cholesterol was thus about 0·3 gram, or 0·1 per cent. The tissues of the worm thus contain cholesterol itself as their most important cholesterol constituent, although some indication of the presence of another crystalline body in small amount was observed.

ECHINODERMATA. ASTEROIDEA. *Asterias rubens*, the starfish. The starfish, which were still living when received, weighed 1,658 grams. They were minced (the juices being collected in plaster of Paris), ground up with sand and plaster of Paris, the mass allowed to dry, and again reduced to powder. After extraction for twenty-one days with ether a pale red-brown extract was obtained, which, on saponification gave a large quantity of a firm porridge-like soap. The red-brown colour of the filtrate was removed on washing with water, leaving the ether solution almost colourless. The crude residue, which was very fluid at 100°, weighed 3·5 grams, and dissolved almost completely in 90 per cent. alcohol. A microscopic examination showed that a mixture of substances was present. Plates, closely resembling those of cholesterol, were seen, together with masses of minute curved needles. After many attempts it was found possible to effect a partial separation in the following way. The crude substance was treated with dry pyridine. A portion proved very insoluble in the cold, but on warming went into solution coming out immediately on cooling as a jelly of minute flexible needles. These were filtered off and treated again with pyridine until they no longer gave the Salkowski test. The pyridine filtrates were collected together and treated as described below.

A. The substance insoluble in pyridine was obtained in silvery flakes, which, after drying in a vacuum, were soft and wax-like to the touch. Heated slowly in a wide capillary tube it melted at 56° to 57° C. It did not give Salkowski's test, and did not absorb bromine in carbon bisulphide solution. In all about 0·2 gram was separated.

On analysis, using a copper boat filled with coarse copper oxide:—

0·0987 gave 0·3014 CO_2 and 0·1301 H_2O
from which C = 83·28 ; H = 14·64 per cent.

These figures are difficult to reconcile with any probable formula. $C_{56}H_{116}O$ requires C = 83·5 ; H = 14·4, but no C_{27} formula agrees at all with the results, which only allow for 2 per cent. of oxygen.

In its properties this body bears a curious resemblance to the little-known higher alcohols which occur in unsaponifiable residues of animal and plant tissues. Among these are cetyl alcohol $C_{16}H_{34}O$, of melting point 50° C., found in spermaceti and the sebaceous glands of geese and ducks; ceryl alcohol, $C_{26}H_{54}O$, of melting point 89° C., and myricyl alcohol, $C_{30}H_{62}O$, melting point 85° C., which are both constituents of beeswax; and chortosterol,[1] $C_{27}H_{54}O$, present to the extent of 0·2 per cent. in the blades and stalks of the grasses.

B. The portion soluble in pyridine apparently contained a member of the cholesterol group since it gave Salkowski's test, and readily absorbed bromine. It was very difficult, however, to separate this from small quantities of A. The substance was thrown out of solution in pyridine by the addition of methyl alcohol, and was then boiled with very dilute ethyl alcohol. The body A then remained undissolved, and the solution was poured off from the oil drops and the process repeated. A small quantity of fairly pure material was finally obtained, which was instantly soluble in a small quantity of pyridine. From dilute alcohol it crystallised in needles, which melted at 143° to 144° C. When treated in chloroform solution with concentrated sulphuric acid, the lower layer first became cherry red, with a green fluorescence, the upper layer slowly becoming of the same colour without the fluorescent green. That the body was not cholesterol was rendered probable by the following experiments. The *acetate*, made in the usual way, crystallised in plates of melting point 128° to 130° C. On heating a portion of the substance with benzoyl chloride for five minutes to 165° C. the melt proved completely soluble in cold absolute alcohol, so that no cholesterol benzoate could have been formed.

In the absence of further investigation it can only be stated that the tissues of the starfish do not, apparently, contain cholesterol, but, instead, two other bodies, one of which may be a hydrocarbon or a complex alcohol, while the other shows some points of resemblance to the members of the cholesterol group.

1. Dorée and Gardner, *Proc. Roy. Soc.*, B. LXXX, 212. Described as Hippocoprosterol by Bondzynski and Humnicki, *Zeit. physiol. Chem.*, XXII, 396.

COELENTERATA. Actinozoa. *Tealia crassicornis* and *Actina equina*, sea anemones.—About two kilograms of moist sea anemone, consisting of the two species above mentioned in about equal proportions, were obtained from the Marine Biological Station at Plymouth. They were ground up with coarse sand, dried in the oven, and the mass extracted for eight days with ether. On saponification of the ethereal extract a considerable quantity of a red, slimy soap was precipitated, which was filtered off and washed with ether. The filtrate, after washing and drying, left a dark brown, unsaponifiable residue, which was dissolved in alcohol and decolourised with animal charcoal. A microscopic examination of the colourless filtrate showed typical crystals of cholesterol, and from the solution crops of crystals, weighing 1·0 gram, were separated. These melted at 143° to 144° C., and after re-crystallisation from acetic ester, at 145° to 146° C., and in all respects appeared identical with cholesterol. That this was the case was confirmed by the following observations and experiments:—

(*a*) Determination of *rotatory power*:

(i) 0·666 gram made up to 25 c.c. with chloroform gave (in mean) − 1·03 in a 1 dm. tube at 20 C. $[\alpha]_D^{20} = -38°$.

The rotatory power of cholesterol for the same concentration[1] would be − 37·3°.

(ii) 0·660 gram made up to 25 c.c. with pure acetic ester gave − 0·73 in a 1 dm. tube at 20 C. $[\alpha]_D^{20} = -27·6°$.

The rotatory power of cholesterol (prepared from brain) in this solvent, is given by Diels and Linn[2] as − 25·6°.

(*b*) *Benzoate*: 0·5 gram of the substance was dissolved in pyridine, and treated with an excess of benzoyl chloride in the usual way. The benzoate obtained was very insoluble in absolute alcohol, from which it crystallised in typical square plates. The re-crystallised product melted at 145° C. to a turbid liquid, which became clear at 175° C., and on cooling showed the colour play of cholesterol benzoate.

(*c*) *Acetate*: 0·3 gram of the substance was acetylated by boiling with acetic anhydride for twenty minutes. The liquid was poured into water, and the precipitate washed and dried in the usual way. The product, after re-crystallisation from acetic ester, melted at 114° to 115° C., and

1. Calculated from Bomer's formula quoted in Lewkowitsch, *Chemistry of the oils, fats and waxes*.
2. *Ber.*, 1908, XLI, 286.

showed opalescent colours on solidifying. It thus agrees exactly with cholesterol acetate, which is the only acetate among the cholesterol group which has been observed to give a colour play on solidification.

(*d*) The purified esters obtained in the above experiments were dissolved in ether and saponified with sodium ethylate. After washing with water, the ether solution, on evaporation, deposited pure white cholesterol, which, after re-crystallisation, melted at 146° C.

(*e*) The pure cholesterol thus obtained was converted to the *dibromide* by the method of Windaus. The liquid set to a crystalline, semi-solid mass, and the dibromide thus formed, after washing and drying in vacuo, melted at 120° C., decomposing a few degrees higher.

An examination of the residues obtained in these experiments, showed no trace of any other crystalline substance. The sea anemone, therefore, contains cholesterol identical in all respects with that of the higher animals.

PORIFERA. [*Suberites domuncula*], *Cliona celata*, *Ephidatia fluviatilis*, sponges. It being thus established that cholesterol, identical in all respects with that contained in the tissues of the Vertebrata, occurs in animals as low down in the scale as the Coelenterata, the investigation of the cholesterol of the sponges becomes of great interest. Many authors have regarded the sponges as being very closely related to the Coelenterata, but apparently the points of resemblance upon which their conclusions were based are superficial only, and the two groups are now considered to be markedly distinct. But when the similarity which exists between the earlier stages of the members of the two groups is taken into account it becomes very probable that both were derived from the Protozoa through a common Metazoan ancestor. But, if the Coelenterata are considerably higher in the scale than the Porifera, the latter undoubtedly stand nearer the Protozoa than any other types of the Metazoa. Formerly, the sponges were regarded as Protozoa, but, 'a sponge is to be regarded as a colony of Protozoa only in the sense in which the same may be said of one of the higher animals. It consists of a complex of cells, some of which have a considerable degree of independence, and some of which have a close resemblance to certain Protozoa; but the same is true of one of the higher animals, the difference being one of degree and not of kind.'[1] So that the Porifera, if not actually members of the lowest group of animals, represent the lowest types available for an investigation such as the present. They are, too, curiously plant-like in the readiness with

1. Parker and Halswell, *Text Book of Zoology*, I, 116. Macmillan, 1897.

which their form becomes modified by external conditions and environment—a peculiarity which is shared by none of the higher animals. So that we have here, possibly, the transition group, from a chemical point of view, between the Coelenterata which contain cholesterol, and the plants which contain phytosterols.

The cholesterol of the sponges was first referred to by Krükenberg,[1] who stated that the red colour of *Suberites domuncula* was due to the presence of a lipochrome, which, under the influence of sunlight, became converted into cholesterol. This remarkable statement was, apparently confirmed by Cotte[2] in the course of his work on the Sponges. In 1904, however, M. Henze[3] re-examined the question, and showed that there was present in *Suberites domuncula* a body of the cholesterol type, to which he gave the name of spongosterol, with a formula $C_{19}H_{32}O$. He also showed conclusively that no relation whatever existed between this substance and the lipochrome of the sponge. Quite recently,[4] Henze has published a further investigation of spongosterol, in which he shows that the body is a definite substance and not a mixture. Attempts to resolve it by the preparation of an acetate dibromide, after the method used by Windaus and Hauth[5] for the separation of phytosterol and stigmasterol, showed that it was not apparently unsaturated like the other cholesterols. Instead of an acetate dibromide a mono-brom-substitution product was formed. Analyses of this and other derivatives led Henze to conclude that spongosterol has the formula $C_{27}H_{48}O$, so that it contains two hydrogen atoms more in the molecule than cholesterol. Its properties, though they bear a general resemblance to those of cholesterol, differ considerably in degree from those of that body. Spongosterol is very insoluble in methyl alcohol, from which it crystallises in plates, many of which have curiously toothed edges. It melts at 123° C, and is laevorotatory, having $[\alpha]_D = -19{\cdot}6°$ C. It gives Salkowski's test in a modified way, but Liebermann's exactly as cholesterol. The acetate melts at 124° C., and the benzoate at 128 °C., the latter becoming a clear liquid at this temperature and showing no play of colours on solidification. When treated with bromine in carbon bisulphide solution it decolourised the bromine, but no definite addition or substitution product could be isolated from the solution. In all these respects spongosterol shows differences from cholesterol, while in some of them it rather

1. *Vergleich. physiol. Studien*, Vol. II, p. 50; Vol. III, p. 3.
2. *Bull. Scientifique de la France et de la Belgique*, XXXVIII, 509.
3. *Zeit. Physiol. Chem.*, 1904, XLI, 109.
4. *Ibid.*, 1908, LV, 427
5. *Ber.*, 1907, XL, 3681.

resembles coprosterol, with which, if Henze's view is correct, it is isomeric. But it may be pointed out that the fact of its forming a mono-brom-acetate when that ester is treated with bromine, instead of an addition product, does not of itself prove the unsaturated nature of the body. For, although cholesterol acetate, when treated with bromine, gives a dibromide, the benzoate on the other hand yields, as shown by Obermüller,[1] a crystalline and very stable mono-brom-derivative $C_{27}H_{44}O.Br.C_7H_5O$ of melting point 136° C. The reactions of the double link, which is situated in the end chain of the cholesterol molecule, undoubtedly present some peculiarities which are not at present capable of explanation. Similar causes may be operative in the case of spongosterol and may mask the presence of an unsaturated linking. Hausmann,[2] and Abderhalden and Le Count,[3] have shown that the anti-haemolytic action of cholesterol is only exhibited so long as the hydroxyl group and the double link are intact. If the latter becomes saturated the anti-toxic power ceases simultaneously. Although in the case of the lower Invertebrata there is no question of cholesterol playing the part of an anti-haemolytic agent, yet this anti-haemolytic action no doubt affords an indication of one at least of its functions in the life processes of cells. As we have seen, all the animal tissues so far examined contain an unsaturated isomer of cholesterol, and, apparently, the case is the same with plant tissues. If the spongosterol of *Suberites domuncula*, therefore, does not contain an unsaturated linking, it would point to the fact that the cholesterol function in this, and if the name be any index of its distribution, in all sponge protoplasm, differs decidedly from that of the cholesterols of other forms of animal and vegetable life.

In order to throw some further light on these questions, and to ascertain whether spongosterol was a constituent common to all sponges, the following experiments were carried out, using, in the first series, the marine species *Cliona celata*. The results obtained show that in this animal there is present apparently one cholesterol body only, which is easily isolated in a state of purity. This new substance, while quite different from spongosterol, bears a remarkably close resemblance both to cholesterol itself and to bombicesterol, with which it is isomeric. For this new cholesterol the name Clionasterol is proposed, as it indicates, in some measure, the source from which the substance is derived.

1. *Zeit. physiol. Chem.*, 1891, XV, 37.
2. *Hofmeister Beiträge*, 1905, VI, 567.
3. *Zeit. Exp. Path. Ther.*, 1905, II, 199.

Investigation of *Cliona celata*

The gamboge-yellow coloured sponges were obtained from Plymouth, and, after very gentle squeezing to remove superfluous water, weighed 1,172 grams. They were minced up, treated with sand and plaster of Paris in the usual way, and the dried mass extracted with ether for ten days. On saponification a small quantity of dark, olive green soap was obtained. The filtered solution, after washing, was a greenish yellow in colour, and on evaporation left 3·2 grams of a bright orange yellow residue, which was just soft at 100° C. This residue dissolved in methyl alcohol, leaving a small quantity of a brownish red pigment, which was practically insoluble in the usual solvents, with the exception of ether. The clionasterol was very difficultly soluble in cold methyl alcohol, crystallising out immediately on cooling. Examined microscopically the crystals appeared in the form of leaves, the base being broad and the smooth edges curving to a point. These leaves were grouped in symmetrical clusters with the pointed ends outward. Occasionally the edges were strongly notched. From the methyl alcohol liquors 2·07 grams of white crystals were isolated (or 0·17 per cent.), and from these, ultimately, 1·2 grams of highly purified substance was obtained.

Clionasterol crystallises from absolute alcohol in needles; from dilute alcohol in plates, some of which closely resemble those of cholesterol, except that they are less regular in shape. It is difficultly soluble in methyl alcohol and in absolute and diluted ethyl alcohol, easily soluble in petroleum ether, acetic ester and acetone. Its melting point is 137° to 138° C., and this remained unaltered after repeated crystallisation. It is laevorotatory, giving figures very near to those of choltesterol itself. Thus 0·442 gram (dried at 100° C.) made up to 25 c.c. with chloroform gave, in a 1 dm. tube at 18° C. (in mean),

$$\alpha = -0{\cdot}655^\circ, \text{ whence } [\alpha]_D^{18} = -37{\cdot}04^\circ.$$

When treated with the usual reagents it shows the following colour reactions: (*a*) When a few drops of concentrated sulphuric acid are added to a solution of the substance in chloroform the upper layer immediately becomes cherry red, and the lower pale yellow with a green fluorescence (Salkowski). (*b*) When strong sulphuric acid is added to a cold saturated solution of the substance in acetic anhydride the acid layer becomes first reddish, then violet blue, and, finally, both layers become green (Liebermann).

To still further characterise this cholesterol, the following derivatives were prepared: —

Clionasterol acetate. 0·5 gram of the substance, mixed with an equal weight of anhydrous sodium acetate, was boiled for twenty minutes with an excess of acetic anhydride, and the liquid poured into water and treated in the usual manner. The acetate is difficultly soluble in alcohol, from which it crystallises in large wide plates of irregular shape, and in methyl alcohol from which it appears in the form of oblong plates, which frequently overlap, forming a saw-like edge. It was moderately soluble in acetic ester, crystallising out in clusters of very long, thin, rectangular plates. The melting point is 133° to 134° C. This substance proved very difficult to burn. A combustion carried out in the ordinary way gave a carbon percentage three units too low. A better result was obtained using a copper boat filled with coarse copper oxide.

0·1319 gave 0·3884 CO_2 and 0·1306 H_2O
C = 80·4 ; H = 11·0
$C_{29}H_{48}O_2$ requires C = 81·3 ; H = 11·2 per cent.

Clionasterol benzoate.—0·6 gram of clionasterol and 0·75 gram of benzoyl chloride were heated together for twenty minutes at a temperature of 165° C. After cooling, the mass was boiled out repeatedly with absolute alcohol, when the benzoate was left as a white crystalline powder. It was almost insoluble in boiling absolute alcohol, and, for analysis, a sample was exhausted with this solvent and then dissolved in acetic ester, from which it crystallised in beautiful glistening leaves, which, under the microscope, appeared as rectangular plates, always longer than wide. In alcohol also it exhibits the same form, whereas cholesterol benzoate crystallises in perfectly square plates from this solvent. Its behaviour on heating was very characteristic, and was carefully observed. Heated in a wide capillary tube it shrinks and begins to soften at 141° C., becoming a turbid, viscous fluid until it melts at 143° to 144° C. to a perfectly clear mobile liquid. If the temperature is allowed to rise to about 160° C., and the tube is then taken from the bath the following play of colours is observed. A pale green first appears, turning to a greenish blue, which then abruptly changes to a deep violet colour, which persists for some time, only gradually fading away. In one or two specimens of the benzoate the violet tint was noticed to change to an emerald green, but these specimens were not, perhaps, so pure as those on which the above observations were made. These colours show slightly during

melting, and may be demonstrated over and over again on the same specimen.

For analysis the benzoate was dried at 100° C. and burnt in a copper boat filled with coarse copper oxide.

0·1527 gave 0·4654 CO_2 and 0·1399 H_2O

	Found	Calculated for $C_{27}H_{45}O.C_7H_5O$
C ...	83·12	83·20
H ...	10·18	10·20

Clionasterol dibromide. 0·35 gram of the substance dissolved in 3·5 c.c. of ether were mixed with 2 c.c. of a solution of bromine in glacial acetic acid, prepared according to the directions given by Windaus.[1] The mixture, after standing in ice, deposited a crystalline precipitate, which, after one hour, was filtered off and washed with dilute acetic acid and water. The filtrate, on the addition of water, deposited more solid matter. The precipitate was dried in vacuo and melted sharply at 114° C., decomposing at 116° to 120° C. A specimen of cholesterol dibromide, prepared in exactly the same way, and melted at the same time, showed a melting point of 123° C. A bromine estimation gave the following figures:—

0·1046 gave 0·0725 AgBr. Found Br = 29·4 per cent.
$C_{27}H_{46}O.Br_2$ requires Br = 29·3 per cent.

Investigation of the Fresh Water Sponge

In view of the discovery thus made that spongosterol is not a constituent of the protoplasm of *Cliona celata*, it was thought advisable to extend the enquiry to a further member of the sponge group, and for this purpose the freshwater sponge was selected. This animal is of great interest for several reasons. From the point of view of this investigation it is, perhaps, the lowest animal type that could be obtained in sufficient bulk for examination. Then, again, it is one of those species which, while indubitably animal, yet possess chlorophyll granules in its tissues so that it can elaborate starch from water and carbon-dioxide by photo-synthetic processes. Although these chlorophyll granules are alien to the animal, being due to infection by a species of alga, their presence tends, so to speak, to form a link between the animal and the plant, and opens up the question whether the plant activities might not determine the formation of a phytosterol in the cells

1. *Ber.*, 1906, XXXIX, 518

of the sponge. On the other hand, we might find the natural cholesterol of the animal side by side with the natural phytosterol of the plant, although the latter should, on account of the relatively small mass of vegetable substance present with the sponge, be inconsiderable in quantity, compared with the former.

Spongilla lacustris and *Ephydatia fluviatilis*, freshwater sponges.—The two common British species of freshwater sponge, though designated *E. fluviatilis* and *S. lacustris*, respectively, commonly occur together in the same situations. The sponges used in these experiments were obtained from the Thames, at Oxford, and, so far as a superficial examination went, consisted chiefly of *E. fluviatilis*. They were only a pale yellowish green, whereas *S. lacustris* is usually a dark green. There is no doubt, however, that both species were present. After removal of twigs and other débris the moist sponges weighed 1,285 grams. When dried the sponge sarcode was left as a dusty powder, which was easily separated from wood, weed and other foreign bodies. It was ground up with sand and extracted for seven days with ether. The extract was a deep green in colour, and after saponification with sodium ethylate, left a considerable quantity of a jelly-like soap, which also was dark green. As it has been stated that fats are not present in most sponges, a portion of this soap was treated with water, in which it was soluble, with the exception of a trace of greenish pigment. After filtering this off and acidifying with hydrochloric acid, a white precipitate was formed, indicating the presence of an organic acid. The green colour was, no doubt, due to chlorophyll, since, on washing the main ether extract, the colour dissolved in the water, leaving the ether solution pale yellow.[1] After drying and evaporating off the ether 7·16 grams of gamboge coloured residue was obtained. This was soluble in alcohol, with the exception of a small, olive green, sticky residue, which was not further examined. The alcoholic filtrate was diluted to about 90 per cent. strength, and a microscopic examination of it showed that two or more substances were present. Large, badly-formed plates were seen, together with rosettes of leaves and thin plates. The two bodies were differently soluble in methyl alcohol, since, on allowing a hot solution of the crude mixture to crystallise on a microscope slide, long, hexagonal crystals first formed, and then, as the solvent evaporated, small plates, somewhat like those of cholesterol, were deposited. Attempts were made to bring about a separation on these lines, but although a substance of melting

1. The chlorophyll is similarly removed on washing the ether extract of grass or of the excrement of the herbivora.

point 135° C. was obtained from the more insoluble part, it was not pure, and the soluble part, which was liquid at 100° C., was obviously a mixture. It was finally discovered that the two constituents were very differently soluble in light petroleum: one, which will be referred to as Spongilla cholesterol A was readily, and the other, Spongilla cholesterol B, almost insoluble in that solvent.

Accordingly 755 grams of fresh sponge was obtained and treated as before, care being taken to remove all foreign vegetable matter. 4·13 grams of unsaponifiable residue were left, which was dissolved in methyl alcohol. On cooling, crops of nearly white crystals, weighing 1·27 gram, were separated, only a very small quantity of crystalline matter remaining in the mother liquors. The crystals were dried, powdered and boiled out several times with petroleum ether. 0·47 gram of spongilla B was left, and to this was subsequently added 0·19 gram, which had passed into solution along with A and was recovered from the filtrate. The two bodies A and B were thus present in approximately equal proportions, 0·66 gram of each being obtained.

Spongilla A was purified by treating the crude substance with cold petroleum ether and filtering from any of B left undissolved. After the separation of B it was dissolved in 90 per cent. alcohol. A drop of the solution examined microscopically showed a large number of small oval-shaped leaves, and on allowing it to crystallise slowly, the substance came out in large glistening leaves or spangles. After purification and drying at 100° C. it melted at 138° to 139° C. It was difficultly soluble in methyl alcohol, from which it crystallised in leaves, closely resembling those of clionasterol, and irregular plates, many of which had curiously toothed edges; moderately soluble in benzene and easily in light petroleum. It gave Salkowski's test exactly as cholesterol, and immediately decolourised bromine in carbon bisulphide solution. It was characterised by the preparation of the following derivatives:—

(i) *The dibromide:* 0·12 gram was dissolved in 2 c.c of ether, and 0·6 c.c. of Windaus' solution of bromine in glacial acetic acid added. In a few minutes the bromine was absorbed and a crystalline precipitate formed, causing the whole mass to become semi-solid. After thorough washing, as previously described, and drying *in vacuo*, the pure white substance melted at 112° to 113° C., turning brown and decomposing violently at 118° C. A qualitative test for bromine was strongly positive.

(ii) *The benzoate:* 0·12 gram of the substance was heated for five

minutes at 165° C. with excess of benzoyl chloride. The melted mass was boiled out several times with absolute alcohol, the nearly pure benzoate being left. This ester was practically insoluble in boiling alcohol. The trace that was dissolved crystallised out, if cooled quickly, in star-like groups of needles: if slowly, in large, irregular plates. After re-crystallisation from acetic ester the benzoate, when heated in a wide capillary tube, began to shrink together at 136° C., and melted at 141° to 142° C. to a turbid liquid, which became clear between 185° and 190° C., and on cooling showed a well-marked play of colours, in the following order: greenish blue, dark blue, deep violet, green (transiently and sometimes not at all), golden brown, finally becoming white. These were observed between glass plates, and were carefully compared with those given by other cholesterol benzoates, as will be described below.

In the characteristic properties of its benzoate and dibromide, spongilla cholesterol A bears a remarkable resemblance to cholesterol itself, and must undoubtedly be regarded as a true animal cholesterol.

Spongilla B was easily obtained pure by extraction with light petroleum, as previously mentioned. It was almost insoluble in methyl alcohol, and went into solution with difficulty even after the addition of a little absolute ethyl alcohol. From this mixture it crystallised in long, well-formed hexagonal plates, which closely resembled those of phytosterol, the two long sides being parallel and the two short ones at each end symmetrically placed, one being longer than the other. In absolute alcohol, it was moderately soluble, crystallising out, on cooling, in glistening leaves, which under the microscope were seen to be of a shorter shape, though generally two opposite corners were truncated, giving the crystals an hexagonal appearance. From acetone it crystallised in needles. After purification it melted at 135° to 136° C., and gave Salkowski's test in the usual way. When treated in carbon bisulphide solution, with a solution of bromine in the same solvent, it absorbed the bromine very slowly and sluggishly. That it was an isomer of cholesterol was shown by the analysis of the benzoate.

The benzoate was prepared in the same way as that of Spongilla A. It also was very insoluble in absolute alcohol, from which it crystallised in large rectangular plates, generally longer than wide, though a few were square. It was difficultly soluble in petroleum ether, but crystallised well from acetic ester.

On heating in a wide capillary tube it melted, showing a bluish fluoresence, at 135° to 136° C., to a turbid liquid which became perfectly

clear and limpid at 139° C. After allowing the temperature to rise to about 160° C., a beautiful and striking display of opalescent colours is observed during cooling. A greenish blue first appears, which immediately turns to reddish violet. This gradually loses the red tint until it has become a bright peacock blue, which slowly passes to a vivid emerald green. The latter colour persists for a long time, and is finally succeeded by a golden brown. These colours are by far the most brilliant of any given by the benzoates of the cholesterol group, and the green colour, which is very characteristic, is given only by this body.

For analysis the benzoate was dried at 100° C. and burnt in a copper boat filled with coarse copper oxide.

0·1573 gave 0·4791 CO_2 and 0·1448 H_2O

		Found	Calculated for $C_{27}H_{45}O.CO.C_6H_5$
C	...	83·07	83·24
H	...	10·22	10·20

Spongilla cholesterol B is thus an isomer of cholesterol with the formula $C_{27}H_{46}O$, and, although the quantity of material available did not permit of the preparation of the dibromide, it was found to absorb bromine, and therefore in all probability possesses a double link in its molecule.

The spongilla cholesterols A and B differ from one another in solubility, in crystalline form and in melting point. The body A absorbs bromine far more readily than B, and the benzoates of the two substances show considerable differences in melting point and colour phenomena. These points are brought out in the following table, in which clionasterol is also included for purposes of comparison.

	Clionasterol	Spongilla A	Spongilla B
Crystalline form { 90% alcohol	Plates	Oval leaves	Oblong plates
Crystalline form { methyl alcohol	Clusters of leaves	Leaves and notched plates	Hexagonal plates
Melting point	137-138°	138-139°	135-136°
Solubility absolute alcohol	Moderately soluble	Moderately soluble	Moderately (hot)
,, petroleum ether	Easily soluble	Easily soluble	Insoluble
Dibromide, melting point	114°	112-113°	—
Benzoate, melting point	141° turbid liquid; clear 143-144° colour play	141-2° turbid liquid; clear 185-190°; colour play	135-6° turbid; clear 139°; colour play

It will be noticed that spongilla B differs markedly from both spongilla A and clionasterol. It bears, however, a certain resemblance to the phytosterols of the cryptogams, which frequently assume the

hexagonal crystalline form (which has not hitherto been noticed among the animal cholesterols). Phytosterol benzoate was found by Ritter[1] to melt at 144° to 145° C. to a clear liquid, and to give a colour play on solidification, in which respect, also, spongilla B resembles it. It would, however, be unjustifiable to attempt to base any conclusion as to the origin of this body on such slight points of agreement, but it is obvious that the relatively large proportion in which spongilla B is present (50 per cent. of the total cholesterols) renders it unlikely that it originates from the algae which live symbiotically with the sponge. The phytosterols of these low plants[2] are believed by Tanret[3] to be very similar to, if not identical with, the fongisterol recently obtained by him in a pure form from spurred rye (*Secale cornutum*). This body has the formula $C_{25}H_{40}O$, melts at 144° C., and gives an acetate melting at 158° C. Spongilla B is certainly dissimilar to this substance. It is also quite possible that spongilla B is characteristic of one species of freshwater sponge, and spongilla A of the other.

There exists, on the other hand, a fairly close agreement in properties between spongilla A and clionasterol. In melting point, solubility and crystalline form they show a great similarity, although the characteristic clusters of leaves in which clionasterol crystallises have not been observed with spongilla A. The mode of formation and melting points of the dibromides agree closely, as do those of the benzoates, except that the turbidity of the clionasterol benzoate clears at 144° C., whereas that of spongilla B persists up to 185° C. A more extended comparison of chemical and physical properties will obviously be required to decide definitely on the relationships of these substances. An attempt was made towards a solution by the following experiments on mixed melting points, equal quantities of each substance being taken in each case.

(*a*) The benzoates of clionasterol and spongilla A.—This mixture melted indefinitely between 135° and 142° C.

(*b*) The benzoates of clionasterol and spongilla B.—This mixture shrank at 136° C., melted to a turbid liquid at 139° C., and cleared at 142° C.

(*c*) The benzoates of spongilla A and spongilla B. This mixture melted completely between 135° and 137° C.

These observations are again inconclusive, as, although it is true the melting points are indefinite, they show no marked deviation from those

1. *Loc. cit.*
2. *Cf.* Gérard, *loc. cit.*
3. *C. R.*, 1908, CXLVII, 75.

of the constituents of the mixture. In this connection, however, it may be mentioned that the admixture of even large proportions of a closely related substance frequently seems to make little impression on the melting point of the members of the cholesterol group. The phytosterol of Calabar beans, which was first obtained by Hesse,[1] of melting point 133°C., has recently been shown by Windaus and Hauth[2] to be a mixture of the pure phytosterol of melting point 137° and stigmasterol, an alcohol with the formula $C_{30}H_{48}O$ and melting point 170° C. The latter body occurs in the mixture to the extent of 20 per cent., and yet only produces a depression of four degrees in the melting point of phytosterol.

The results of this examination of the sponges make it very probable that each species of sponge is characterised by a different member of the cholesterol group, so that further investigations may bring to light a variety of animal cholesterols corresponding with those already known as constituents of plants.

SUMMARY AND DISCUSSION OF RESULTS.

1. The results obtained for the distribution of cholesterol and its allies in the animal series may be summed up as follows:—

CHORDATA	Cholesterol is universally present and is not accompanied by any closely related body.
MOLLUSCA—	Cholesterol is present.
ARTHROPODA—	
CRUSTACEA	Cholesterol is present.
INSECTA	Cholesterol may occur in *Bombyx mori*, of which, however, the chief constituent is bombicesterol, an analogue of cholesterol. In *Blatta orientalis* a body of the cholesterol type is present.
ANNULATA—	Cholesterol is present.
ECHINODERMATA	*Asterias rubens* does not contain cholesterol, although a body of a similar type is present.
COELENTERATA—	Cholesterol is present.

1. *Liebig Annalen* CXCII, 175.
2. *Ber.*, 1906, XXXIX, 4378.

PORIFERA *Suberites domuncula* contains spongosterol, which, although probably related to cholesterol, shows marked differences in properties (Henze). *Cliona celata* contains clionasterol, and the fresh water sponge contains apparently two bodies, all of which bear the closest resemblance to cholesterol.

2. In the following table the quantitative results obtained are collected together.

Animal	Weight in grams	Weight of dried non-saponifiable matter	Per cent.	Weight of Cholesterol obtained in grams	Per cent.
Rabbit	2800	6·0	0·21	3·24	0·117
Grass Snake	246	0·44	0·18	0·20	0·08
Mackerel	1452	1·27	0·09	0·32	0·022
Whelk	1179	4·50	0·38	1·46	0·124
Crab	538	1·0	0·19	0·25	0·048
Cockroach	194	0·5	0·25	—	—
Lob Worm	286	0·95	0·32	0·31	0·10
Starfish	1658	3·5	0·21	—	—
Sea anemone	2000 ?	—	—	1·5	0·07 ?
Cliona	1172	3·20	0·27	2·07*	0·17
Spongilla	755	4·13	0·54	1·027*	0·17

* Not cholesterol itself.

3. These results show that the protoplasm of all the animals examined, and, therefore, probably of all animals, contains at least one member of the cholesterol group. This member is not always cholesterol itself, although it has now been proved that this substance is very widely distributed throughout the animal kingdom. In the warm-blooded Vertebrata it is universally present, and is the only body of its type that has ever been observed in them. The structures of the cold-blooded Vertebrata have also been found to contain cholesterol. In the Invertebrata cholesterol has been recognised as widely, but not uniformly, distributed throughout the series. In the highly organised Mollusca it occurs in comparatively large quantity. In the phylum Arthropoda the Crustacea contain it, whereas in Insecta its place is taken by nearly allied substances. In Annulata it is present, but not apparently in Echinodermata. Its occurrence, again, in the Coelenterata is significant as showing that the cholesterol function in the protoplasm of these low animals can be carried out by the same substance as in the case of the most highly developed

types. Cholesterol itself is not present in the Porifera, and, unfortunately, no Protozoan species could be obtained in quantity sufficient for examination.

4. In those species in which cholesterol itself was not found, there was present in each case as a substitute a body of very similiar properties. The cholesterol constituent of *Asterias rubens*, representing Echinodermata, was not very exactly defined, owing to difficulties which were met with in effecting its separation from other non-saponifiable substances. It appeared, however, to be similar to cholesterol in its properties: it was unsaturated and gave Salkowski's test. The researches of Menozzi and Moreschi on *Bombyx mori*, and the work described in this paper on the Porifera, show that the place of cholesterol in these animals is filled by previously unknown members of the cholesterol group, which in all their properties are remarkably similar to cholesterol itself. The physical and chemical properties of cholesterol, of bombicesterol, of clionasterol, and of the cholesterols of spongilla form a complete parallel. They are all alcohols of the formula $C_{27}H_{46}O$, contain one well-defined double link in the molecule, and agree in the behaviour of their derivatives, notably in the solubility and fusion phenomena shown by their benzoates. They differ from one another chiefly in their crystalline form and the melting points of their acetates and dibromides. Among these, also, no sign of an evolution (if one may use the term) of cholesterol is apparent. Just as the cholesterol of man is present in the sea anemone, so the bombicesterol of the highly organised insect is exactly matched by the clionasterol of the sponge.

5. With the possible exception of the spongosterol of *Suberites domuncula*, to which reference has already been made, all the animal cholesterols so far examined are isomeric with and similar in properties to cholesterol itself. The unsaturated linking and the hydroxyl group, which Hausmann and others have proved to be essential to the performance of the anti-toxic function of cholesterol, are apparent in all of them. It may not, therefore, be too much to say that the protoplasm of all animals contains as one of its essential constituents a cholesterol; that is to say, a singly unsaturated, monatomic alcohol, isomeric with or very closely related to cholesterol itself.

6. Can this generalisation be applied to all protoplasm, vegetable as well as animal? Our knowledge of the vegetable cholesterols is based upon a comparatively large number of investigations, which are not, however, as a rule, of a very precise character. In an Inaugural

Dissertation[1] Hauth has given a list of the various phytosterols which have been described in the literature under various names. Of these thirty-one have melting points lying between 132° and 137° C., and closely resemble cholesterol. They were all obtained directly or indirectly from the Phanerogams. The work of Windaus and Hauth[2] has made it probable that all of these bodies are mixtures of phytosterol with other alcohols which are capable of forming isomorphous mixtures with it, and even when present in considerable proportion do not materially lower the melting point of phytosterol. Their separation is extremely difficult, but Windaus and Hauth succeeded in bringing it about in the case of the phytosterol of Hesse originally obtained from Calabar beans. This substance was found to contain 20 per cent. of an alcohol, stigmasterol, of melting point 170° C.; the residue consisted of phytosterol which had previously been obtained pure by Burian[3] from wheat germ. This latter body would, therefore, appear to be typical of the higher plants, just as cholesterol is of the higher animals. It is an isomer of cholesterol, with the formula $C_{27}H_{46}O$, and possesses a well-marked double link. It is very similar in properties to cholesterol, and in all probability possesses a similar structure in the side chain.[4]

Our information with regard to the cholesterols of the lower plants is due chiefly to the researches of Gérard[5] and Tanret.[6] The former showed that in yeast and some species of lichen there occurred a body of melting point 136° C., which resembled the phytosterols in some respects, but differed from them in possessing a very high laevorotatory power − 105°. A similar substance was obtained from *Staphylococcus alba* and *Fucus crispus*. These bodies show a decided resemblance to the ergosterol of Tanret,[7] which was first obtained by him from *Secale cornutum*, and which he has recently[8] succeeded in separating in a pure form. It is accompanied apparently in spurred rye (*Secale cornutum*) by fongisterol, which, in the opinion of Tanret, is a lower homologue of ergosterol with the formula $C_{25}H_{42}O$. Ergosterol is apparently isomeric with cholesterol. It melts at 165° C., and has a rotatory power in ether of − 105·5°.

A large number of other phytosterols have also been described, but practically nothing is known of their nature, and in all probability they

1. *Zur Kenntnis der Phytosterine*. Freiburg i. B. 1907.
2. *Ber.* 1906, XXXIX, 4378.
3. Under the name of sitosterol, *Monatshefte*, XVIII, 553.
4. Pickard and Yates, *J.C.S.*, 1908, XCIII, 1928.

5 & 6. *Comptes rendus*, 114, 1544; 1898, 909. Also *Jour. Phar.* 1895 (6), 1, 601.

7. *Ann. de Ch. et de Phys.*, XX, 289.
8. *Comptes rendus*, 1908, CXLVII, 75.

are not single substances. It will be apparent, however, that in the protoplasm of plants, as well as in that of animals, a 'cholesterol' appears to be universally present.

7. The following scheme embodies the results obtained by Hausmann (*loc. cit.*) in his investigation of the anti-haemolytic power of various members of the cholesterol group and their derivatives. It will be noticed that the naturally occurring alcohols, cholesterol, phytosterol and parasitosterol, all have a marked anti-haemolytic power. If the hydroxyl group be replaced by chlorine (as in cholesteryl chloride), or by hydrogen (as in cholestene), or be esterified (as in phytosterol propionate), the activity entirely disappears, although the unsaturated linking remains unaltered. If the double bond be saturated, as in cholesterol dichloride, or be modified by ring formation, as in the case of cholestanonol and probably coprosterol, the anti-haemolytic power is reduced to a minimum although the hydroxyl group is still present. Spongosterol is described as having a feeble anti-toxic power, and as being similar to coprosterol in this respect—observations which are in agreement with the observed chemical reactions of these bodies.

	Formula	Anti-haemolytic function towards saponin
Cholesterol	$C_{27}H_{45}.OH$	Active
Cholesteryl chloride	$C_{27}H_{45}.Cl$	None
Cholestene	$C_{27}H_{46}$	,,
Cholesterol acetate	$C_{22}H_{45}.O\,C_2H_3O$	,,
Cholesterol dichloride	$C_{27}H_{45}\langle^{OH}_{Cl_2}$	Doubtful
Coprosterol	$C_{27}H_{47}.OH$	Feeble
Cholestanonol	$C_{27}H_{46}O_2$	Feeble
Phytosterol	$C_{27}H_{45}OH$	Active
Phytosterol propionate	$C_{27}H_{45}O.C_3H_5O$	None
Parasitosterol	$C_{27}H_{45}.OH$	Active
Spongosterol	$C_{27}H_{47}.OH$	Feeble

8. In the following table are collected together, for purposes of reference and comparison, the properties of the best known members of the cholesterol group which are isomeric with cholesterol, and, like it, unsaturated. Spongosterol, though its similarity in these respects to cholesterol is doubtful, is also included.

	M.p.	$[\alpha]_D$*	Acetate M.p.	Benzoate M.p.	Dibromide M.p.	
CHOLESTEROL	147°	−37°	114°	145° (colour play)	123°	Generally distributed in the animal kingdom.
BOMBISCESTEROL	148°	−35°	114°	146°	111°	In *Bombyx mori*.
CLIONASTEROL	138°	−37°	133°	143° (colour play)	114°	In *Cliona celata*.
SPONGILLA A	138°	—	—	142° (colour play)	113°	In freshwater sponge.
SPONGILLA B	136	—	—	136 (colour play)	—	,, ,, ,,
SPONGOSTEROL	123	−19	124°	128°	—	In *Suberites domuncula*.
PHYTOSTEROL	137°	−34°†	127°	145° (colour play)	98°	In the Phanerogams.
ERGOSTEROL	165°	−126°	180°			In the Cryptogams.

* In chloroform solution. † In ether solution.

9. The present work has not made clearer the obscure question of the origin of cholesterol in the animal organism. It is certainly not without significance that cholesterol never occurs among plant tissues, and when the great difference in the nutrition of the animal and the plant is considered, the conclusion seems almost justifiable that the cholesterol of the animal is derived from the phytosterol of the plant. For the plant, in virtue of its power of photo-synthesis, can elaborate starch from carbon dioxide and water, and protein from the carbohydrate thus formed and the nitrogenous food stuffs absorbed from its medium, while the animal can only utilise the protein, carbohydrate and fat which has been previously built up from inorganic material by the plant. To these purely nutritious substances may possibly be added phytosterol elaborated by the plant. Herbivorous animals, whose tissues and fluids contain cholesterol, eat only phytosterols; and it has been shown that if fed on diets free from all members of the cholesterol group, the rabbit, for example, can absorb cholesterol when given with the food.[1] That it can absorb and utilise phytosterol as such has not yet been experimentally proved, but since it can and does absorb cholesterol, it is obvious that such absorbed cholesterol is required normally by the organism, and must usually be derived from the vegetable cholesterols taken with the food.

1. Dorée and Gardner, 'The Origin and Destiny of Cholesterol in the Animal Organism,' Part III, *loc. cit.*

In conclusion, I wish to thank Professor E. A. Minchin for some kind suggestions with regard to the work on sponges, and the Director of the Marine Biological Station at Plymouth for obtaining for me the various marine species used in the research.

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ALLYL ISOTHIOCYANATE: SOME ASPECTS OF ITS PHYSIOLOGICAL ACTION

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There are but two allyl compounds in general use—the sulphide and the isothiocyanate, and with the latter this communication chiefly deals, the former having already furnished the theme of a previous research.[1]

Allyl isothiocyanate, or oil of mustard, is a well-known rubefacient and counter-irritant when applied to the skin, and in addition is in small doses in constant use internally as a condiment; it appeared, therefore, desirable to ascertain in what directions its action resembled or differed from that of its ally, and with this intent a number of experiments were undertaken upon deeply etherised rabbits, the methods followed being precisely the same as those indicated in the previous paper.

In the first instance one minim (0·06 c.c.) of the pure synthesised drug was injected into the jugular vein of a large animal, with the result that it died in convulsions a few minutes later, thereby demonstrating how much more deadly the isothiocyanate is than the sulphide, and the necessity of finding some bland menstruum which would mix with it in all proportions and form a solution of constant and uniform strength throughout. Olive oil proved to be the most suitable vehicle for the purpose, because when injected in small quantity into a vein it exhibits no physiological action either on the heart, blood pressure or respiration, which were the main effects to be attended to in the present research.

Ten per cent. and 20 per cent. solutions of oil of mustard in olive oil were accordingly prepared. For the purposes of this report it will be sufficient to give in detail the stages in one typical case and to comment upon others as occasion arises.

In a rabbit weighing 2,200 grammes, and deeply anaesthetised, 1½ minims (0·09 c.c.) of a 20 per cent. solution of allyl isothiocyanate in olive oil were injected into the jugular vein in six seconds, the initial pressure in whose carotid artery was 72 mm. Hg, with the result that the pressure began to rise slightly at first, reaching 73 mm. in seven seconds;

1. *Bio-Chemical Journal*, Vol. II, pp. 325-339.

this is immediately followed by a rapid fall, reaching its lowest point at 28 mm., twenty-seven seconds after the commencement of the injection; a slow rise in pressure follows to 45 mm., which was reached at the sixty-seventh second, to be followed by a gradual fall to 39 mm. at the 127th second, when it again began to rise very slowly, only reaching 70 mm. at the 387th second, after which it again sank almost imperceptibly to 53 mm. at the 950th second, at which moment the record ceases. (Fig. 1.)

Allyl isothiocyanate, therefore, exerts its maximum depressor effect almost immediately after administration, followed by rapid partial recovery; this is succeeded in turn by another fall of blood pressure of less intensity but of longer duration, and, like its predecessor, followed by a slower rise, but only to fall again, and so on. If sufficient time is given for the drug to be eliminated, chiefly by the lungs, these pressure waves die out and the normal blood pressure is restored.

This drug, like its congener, has a powerful effect upon the respiratory system, which lasts for a considerable time, but the disturbance caused by it is more rapidly recovered from than that produced in the blood pressure.

At first the respiratory movements are diminished in number and in amplitude, but is soon followed by increased frequence, with further diminution, the animal's chest becoming inflated owing to the inspiratory efforts greatly exceeding the expiratory, all the extraordinary inspiratory muscles being brought into play; the tracing, therefore, sinks rapidly and somewhat suddenly at the twelfth second after the commencement of the injection. Soon the respiratory efforts, though still rapid, increase in amplitude, becoming now and again convulsive, despite which they show a tendency towards recovery from the fifty-seventh second, which practically corresponds to the time at which the carotid curve is nearing its first pressure maximum. Thereafter the movements become less extensive and less frequent and finally attain their normal rate and magnitude about the 800th second after injection.

Allyl isothiocyanate, like allyl sulphide, produces a certain measure of immunity in the animal after it has recovered from a first dose, and therefore 3 minims (0·18 c.c.) of the same solution was administered in seven seconds by injection into the jugular vein of the same animal after it had completely recovered from the effects of the first, i.e., after half-an-hour, complete anaesthesia beng maintained during the whole time.

The carotid pressure at the moment of injection was 78 mm. Hg, but before the injection was over a slight fall in the tracing was observed, which was followed at the thirteenth second by a more rapid fall that

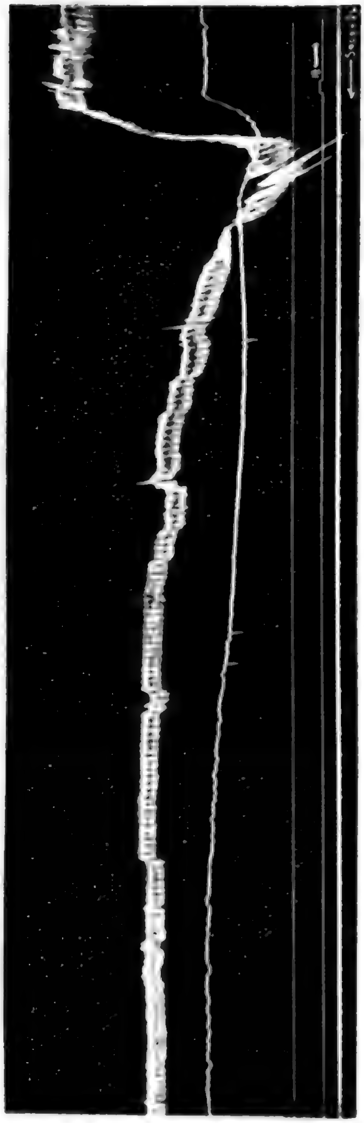


Fig. 1. Effects of injection into the jugular vein of 1·5 minims (0·09 c.c.) of a 20 % solution of allyl isothiocyanate in olive oil.

Fig. 2. Effects of injection into the jugular vein of a rabbit of 3 minims (0·18 c.c.) of a 20 % solution of allyl isothiocyanate in olive oil.

reached a minimum of 55 mm. Hg at the twenty-sixth second. From that moment a rapid rise commenced, crossing the normal line at the thirty-fifth second, and continuing in a series of humped curves to a maximum of 98 mm. at the seventy-second second, thereby attaining a height of 20 mm. above the normal. (Such a rise never occurs with a first dose.) This rise is followed by a very slow fall to 92 mm. at the 290th second, at which point there was a marked change in the respiration, accompanied by an abrupt fall in pressure to 83 mm. With the exception of a slight rise followed by a slight fall, the pressure gradually sank to 82 mm. at the 800th second, reaching the normal as the effects of the drug slowly passed away. (Fig. 2.)

With this dose, which would have been fatal to a fresh rabbit, the breathing became quicker and shorter from the moment the carotid pressure commenced to fall, the chest very rapidly became inflated and the tracing falling almost suddenly, the respiratory movements increasing in amplitude, despite their rapidity, as the bottom of the curve was reached; the tracing then rose somewhat, but at the fiftieth second spasms moderate in force and duration supervened, and were succeeded by a few small respiratory efforts that quickly grew in amplitude, the respiration finally settling down to a steady and regular rhythm, about three times less rapid than before the injection, passing into normal about the 320th second.

The animal having again recovered, a dose of 6 minims (0·36 c.c.) was administered in ten seconds in the same manner as before. This produced a fall of 7 mm. in the pressure curve in twenty seconds, followed by a rise to 16 mm. above the initial pressure some eighty-five seconds after the injection, from which point it began to sink until the animal died.

On the respiratory mechanism it produced first a quickening of the breathing with diminished amplitude, and at the twentieth second the usual sudden fall in the trace occurred followed by convulsions, in which the whole body participated. Tremors first appear in the abdomen and thorax, then in the hind limbs, which become rigid, with the toes widely separated and fully extended; the muscles of the spinal column are next attacked and lift the animal quite off the table in rigid opisthotinos, finally the fore limbs become affected, but the spasm in them is less marked.

At this stage artificial respiration was had recourse to, but some little time elapsed before the chest became sufficiently flaccid for it to be effective. During the whole spasm the heart was beating strongly, and the blood pressure kept up, reaching its highest point just after artificial respiration became effective, at which time the spasm also began to subside

in the hind limbs, though it continued in the paws and passed to the head and neck, producing convulsive movements of the jaw muscles and wheel-like movements of the eyeballs.

The animal was now killed by stopping the respiratory pump and opening the thorax, having been kept in the deepest anaesthesia during the whole course of the experiment.

The heart when exposed was beating well, the drug apparently in no way affected its muscle fibres, though this large dose of the drug produced violent and convulsive contractions of the skeletal muscles, the extensors being chiefly affected by it.

Allyl isothiocyanate is, therefore, a much more powerful poison than allyl sulphide. It attacks the respiratory system more powerfully than the vaso-motor, though its effects pass off more quickly from the former than from the latter. Poisonous doses kill by paralysing the respiratory centre, as with allyl sulphide. The expiratory centre seems more affected than the inspiratory, resulting in considerable chest inflation.

A tolerance is set up for the drug on the administration of repeated doses of the same strength, so that a stronger dose has to be given each time to produce an equal effect. By the first dose the vaso-motor centre is depressed and recovers but slowly, but from subsequent doses it not only recovers more quickly, but is less affected and becomes stimulated, so that the blood pressure rises high above the normal. This may be due partly to the muscular tremors that invariably occur with moderate doses, causing a more rapid return of the blood from the compressed veins towards the heart, but that is not the only cause, for considerable rises in pressure can be obtained by administering repeated small doses that do not produce visible tremors. With small doses the depressor effect alone is marked, with larger ones a pressor effect is subsequently developed, and with large, i.e., fatal doses, a pressor effect alone may be registered. The vaso-motor centre seems more sensitive than the respiratory centre to small doses, though with medium and large doses the reverse is the rule. (Fig. 3.)

With a small dose the respirations may be only quickened and somewhat reduced in excursion without any chest inflation, which is such a constant occurrence with medium ones.

The minimum fatal dose was not accurately determined, but it approximates to 1 minim (0·06 c.c.) of a 20 per cent. solution per kilo. of body weight, i.e., one-fifth minim (0·012 c.c.) of normal oil, and therefore this drug is two and a half times more fatal than allyl sulphide.

Even with fatal doses, if the animal be kept alive by artificial respiration, the vaso-motor system remains efficient, because every

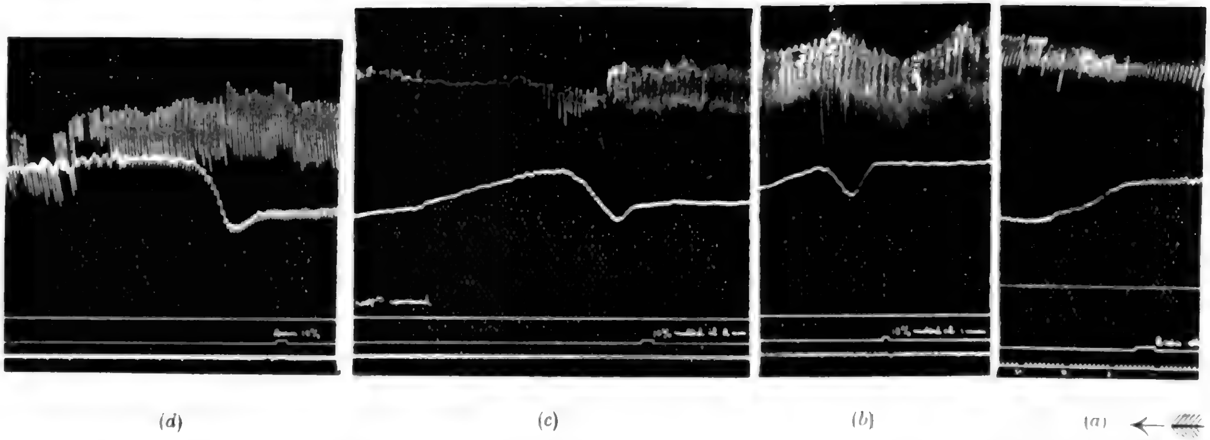


FIG. 3. Showing the effect of repeated small doses of 10% allyl isothiocyanate on the carotid pressure of the same animal

(a) Depressor effect alone marked.
(b) Depressor and pressor effects about equal.
(c) Depressor effect far surpassed by subsequent pressor effect
(d) Depressor effect slight, with subsequent great and sustained pressor effect.

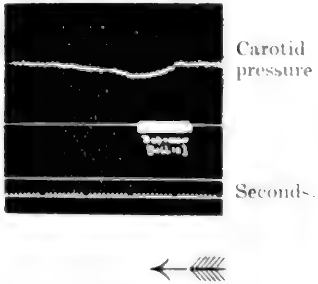


FIG. 4. Effect on the carotid pressure of stimulating the depressor nerve in a rabbit that has been injected with a strong dose of allyl isothiocyanate.

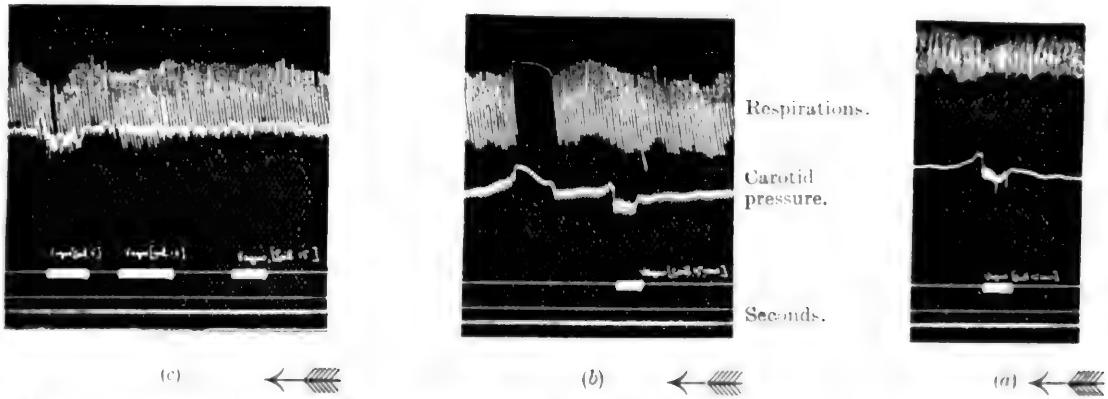


FIG. 5. Effect on the carotid pressure of stimulation of the peripheral end of the divided vagus, as the dose of the drug is increased.

(a) 1 mm. = 0·06 c.c. 10% allyl isothiocyanate.
(b) 3 mm. = 0·18 c.c. 10% allyl isothiocyanate.
(c) 7 mm. = 0·42 c.c. 10% allyl isothiocyanate.

cessation of the ventilation is followed immediately by a rise in blood pressure, which passes off immediately the air-pump is restarted, and because when the depressor nerve was stimulated electrically in the only case in which its efficiency was tested, there was a slow fall of pressure succeeded by a slow rise after cessation of its excitation. (Fig. 4.)

With the vagus nerve things are different, it loses its efficiency upon the heart as the strength of the dose increases, failing to reduce the blood pressure, though it can still impede the heart beat to some extent; in this the action of the isothiocyanate closely resembles that of allyl sulphide. (Fig. 5.)

On skeletal muscles the drug acts powerfully, producing spasm, the extensor groups being more affected than the flexor. Its action in small doses on cardiac muscles is slight and of short duration; beginning early, it passes off in about 150 seconds and amounts to no more than a slight decrease in the rate of the heart beats, with lengthening of the systole.

With the frog's heart phenomena occasionally seen with allyl sulphide are well marked with the isothiocyanate, more especially so with winter frogs. In all cases pithed and decerebrated frogs were used, and the records obtained by placing the frog on a Pembry myograph, exposing the heart, inserting a hook in the apex of the ventricle, from which a thread passed to the lever of the apparatus. The drug, either pure or diluted with olive oil, was passed directly into an auricle by means of a hypodermic syringe.

A dose of 1 minim (0·06 c.c.) of the pure drug stops the heart at once, but a dose of half that quantity only takes effect after thirty seconds, the ventricle muscle passing into delirium before finally ceasing to beat. The auricles, however, continued to beat synchronously, though jerkily, for a considerable time longer.

One minim (0·06 c.c.) of a 33 per cent. solution produces some diminution both in amplitude and rate, followed suddenly some sixty seconds later by a marked change in speed with increase of amplitude, which gradually declines as the speed again increases for a while This is followed by marked slowing just before the sudden arrest of ventricle comes on, some 280 seconds after the injection. The auricles beat normally throughout, and continue so doing for another seventy seconds at least, after which their speed very gradually diminishes. Though arrested, the ventricle is not permanently stopped but resumes beating again with occasional stops, that become shorter as time wears on, until, by the end of fifteen minutes, the whole heart so far recovers as to beat quite rhythmically though slowly and with diminished force.

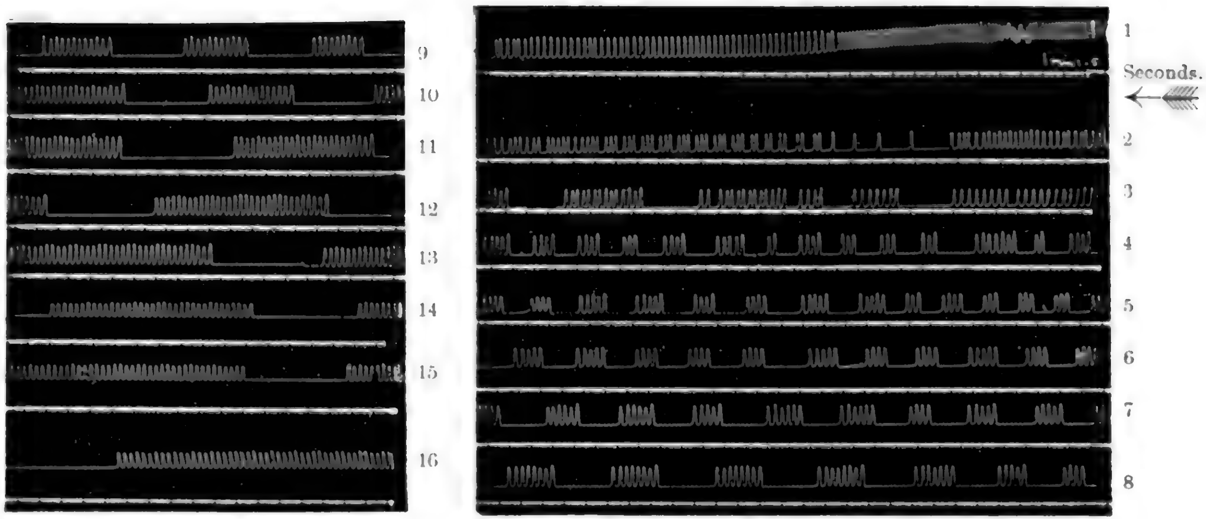
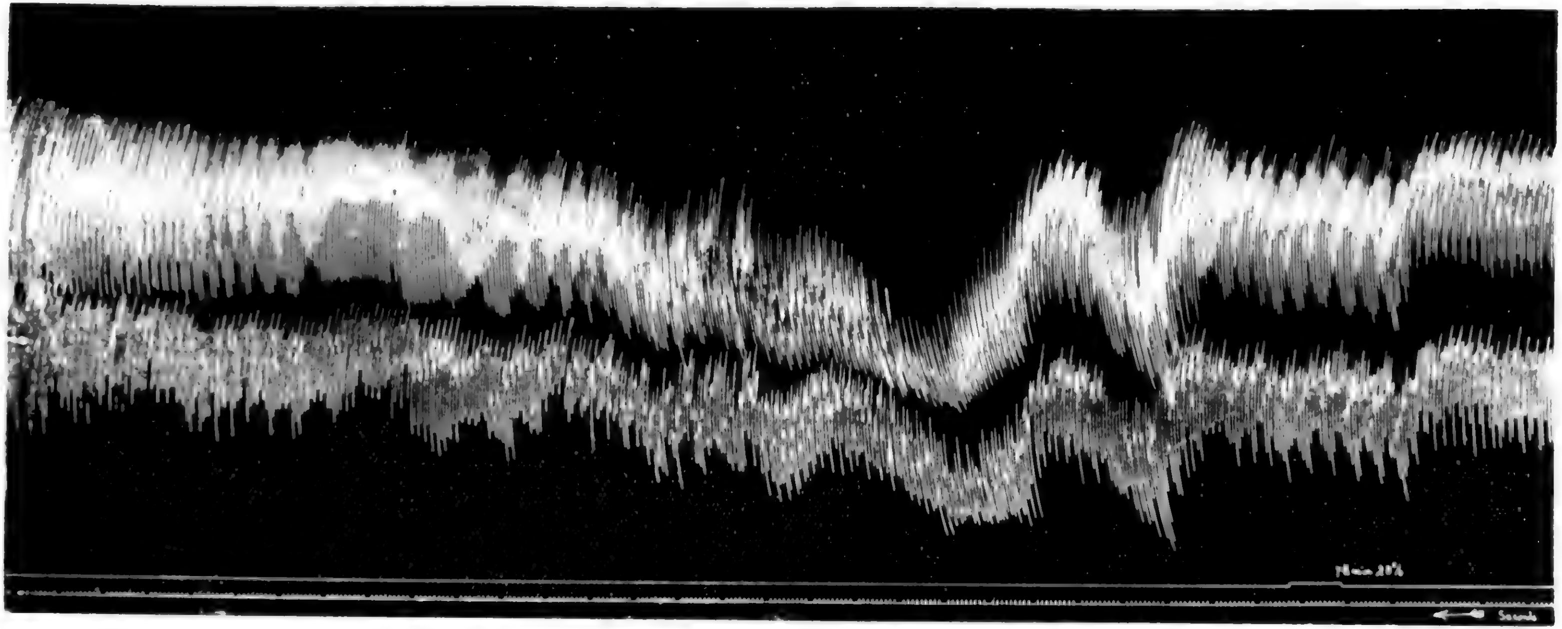


FIG. 6. Showing the effect produced upon the frog's heart by injection into one auricle of 1 minim = 0·06 c.c. of a 20 % allyl isothiocyanate solution. The phenomenon of heart block is clearly shown, and also the relative durations of the periods showing blocking and those free from it.



[illegible] 7 [illegible] of 7 [illegible] of a 20% solution of allyl isothiocyanate into the jugular vein of a rabbit, [illegible] the experiment. Levers (writing over pulleys) were attached by hooks, the upper in the auricle, and [illegible]

With this dose the results naturally vary somewhat with the size and vigour of the frog used; with small weak frogs the heart is permanently arrested, the auricles always outlasting the ventricle for a considerable time; with large and vigorous frogs partial or complete recovery in the rhythmic sequence is customary; the speed, however, seems always permanently diminished.

I have obtained the best results with an injection of 1 minim (0·06 c.c.) of a 20 per cent. solution into the auricle, as may be seen from the tracing here given, in which the production of heart block, with gradual recovery therefrom, is better illustrated than is possible by mere description. (Fig. 6.)

When frogs, taken fresh from the fields in summer, are treated as above, heart block rarely supervenes, the heart either stopping suddenly as a whole with large doses, or becoming gradually slower and feebler with smaller ones, until it finally ceases to beat altogether.

The effect of the drug upon the mammalian heart may be studied in the rabbit, provided artificial respiration is had recourse to, because the large doses required are very much more than sufficient to paralyse the respiratory mechanism. In all cases the ventricles are more profoundly affected than the auricles; with a dose of 7½ minims (0·45 c.c.) of a 20 per cent. solution injected into the jugular vein the amplitude of the heart beat is first lessened, this is followed by diminished speed with some increase in amplitude, but the heart is finally paralysed in about twenty minutes. (Fig. 7.) Only in one case was some blocking noticed, towards the end of the experiment and after the animal had received several consecutive and increasingly larger doses of the drug. In this case three auricular beats corresponded with one of the ventricles. With these large doses the auricles occasionally fail a few seconds before the ventricles, but this is not the general rule.

Conclusion

Allyl sulphide and allyl isothiocyanate act in a similar manner upon the organism, the latter drug being the more powerful; both paralyse the respiratory and vaso-motor centres, both produce muscular spasms and affect the heart beat, and both lower the body temperature, as most essential oils do. Neither can be recommended for internal administration, despite the fact that they are commonly taken in minute quantity with food.

CHOLINE IN ANIMAL TISSUES AND FLUIDS

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Introductory and Historical

Although the morphological changes accompanying nerve degeneration have been very minutely studied and certain definite results obtained, the study of the corresponding chemical changes has not, so far, thrown any light on the metabolism of the nervous tissues. Gulewitsch[1] was the first to isolate free choline, one of the substances entering into the constitution of nerve tissue, from extracts of ox-brain. Vincent and Cramer[2] isolated very small amounts of a similar substance by a process less likely to produce decomposition. These observations constitute the only direct chemical evidence of metabolic processes in nervous tissues. But it must be pointed out that the possibility of *post-mortem* changes, or of changes occurring during the manipulations involved in the isolation, cannot be altogether excluded. This latter objection seems, however, to lose its weight in view of the observations of Gumprecht,[3] who found by a micro-chemical test that a substance giving the reactions of choline is present in the normal cerebro-spinal fluid of healthy animals and of patients suffering from diseases other than nervous.

Another fact pointing to the existence of metabolic processes in nervous tissue has been brought forward by Waller,[4] who suggested that the increase in electrical response after repeated excitation of nerve is due to carbonic acid having been evolved in consequence of the activity of the nerve.

The evidence put forward by Halliburton[5] for the presence of choline

1. *Zeitschr. f. physiol. Chem.*, XXIV, s. 513, 1898.
2. *Journ. of Physiol.*, Vol. XXX, p. 149, 1904.
3. *Verhandl. d. Congress f. innere Med. Wiesbaden*, s. 326, 1900.
4. *Centralbl. f. Physiol.*, XII, s. 745, 1899.
5. *Journ. of Physiol.*, Vol. XXVI, 1900-1, and numerous other publications.

in saline extracts of nervous tissue can not, in our opinion, be accepted. He believes that the effect on the blood pressure of nervous tissue extracts, observed by himself and by Osborne and Vincent,[6] is due to the presence of choline, and that by means of this 'Physiological test' the presence of choline in animal extracts and fluids can be detected. This was disputed by Osborne and Vincent,[7] Vincent and Sheen,[8] and Vincent and Cramer,[9] who pointed out the difference in behaviour between brain extracts and choline after the administration of atropine, and demonstrated that the depressor effects are due to substances other than choline.

The chemical evidence submitted by Halliburton, which is based on the appearance of crystals of a double salt of platinum chloride in brain extracts, has been shown to be unsound. Vincent and Cramer, and independently Mansfeld,[10] showed that these crystals consist almost entirely of inorganic salts, from which it is impossible to free the extracts even after repeated extraction with absolute alcohol. These results were confirmed by French and Allen;[11] and Bayliss [12] in his investigations on adsorption, showed that this phenomenon is due to the adsorption of inorganic electrolytes by organic colloids.

The chemical evidence as to changes in pathological conditions of the nervous system is of a more definite character. Mott and Barrat[13] were the first to show that degeneration in the spinal cord leads to a diminution of the phosphorus contents. Noll[14] found that the protagon diminishes after nerve section, and Halliburton[15] also showed that a diminution of phosphorus occurs under these conditions.

These results led Mott and Halliburton[16] to seek for choline in the blood in conditions of nerve degeneration. In a series of experiments on cats the sciatic nerves were divided, and the physiological and chemical tests for choline were applied to the blood of these animals. The results showed a striking parallelism between the effect on the blood pressure and

6. *Journ. of Physiol.*, Vol. XXV, 1900.
7. *Loc. cit.*
8. *Journ. of Physiol.*, XXIX, p. 242, 1903.
9. *Loc. cit.*
10. *Zeitschr. f. physiol. Chem.*, Bd. XLII, 1904.
11. *Proc. Physiol. Soc.*, p. 29, 1903; *Journ. of Physiol.*, p. 30.
12. *Bio-Chemical Journ.*, Vol. I, p. 209, 1906.
13. *Proc. Physiol. Soc.*, p. 3; *Journ. of Physiol.*, Vol. XXIV, 1899.
14. *Zeitschr. f. physiol. Chem.*, XXVII, p. 370, 1899.
15. *Croonian Lectures*, 1901.
16. *Proc. Physiol. Soc.*, Feb., 1898; *Journ. of Physiol.*, Vols. XXI, XXII, and XXIV, Feb., 1898.

the number of crystals obtained. These results constitute the only experimental basis for the so-called 'Choline hypothesis,' that degenerative processes in nervous tissues can be detected by the appearance of choline in the blood. Although it is now generally admitted that the tests employed in these experiments are fallacious, the question has not, up to the present, been re-investigated in experiments in which more reliable tests for choline have been employed. Such tests are recommended by Gumprecht,[17] French and Allen,[18] Donath,[19] and Rosenheim,[20] but have only been applied to the cerebro-spinal fluid in the case of patients suffering from nervous diseases. With the exception of Gumprecht's observations, no controls have been made upon patients suffering from other kinds of diseases, although the facts given above as to the presence of a substance giving the reactions for choline in normal cerebro-spinal fluid would appear to have rendered some such control imperative. The attempt to find choline in the blood in cases of nerve degeneration has now been almost completely abandoned. The results of clinical investigation by various observers are very conflicting, and are explained by each author as being due to the inferiority of the tests employed by the others.

Experimental

In view of the conflicting nature of the evidence before us, it seemed to me very desirable to test the choline hypothesis by means of a series of experimental lesions in animals. My observations have so far been restricted to testing for choline in the blood of those animals where choline is normally not present. The fact that choline is found in normal cerebro-spinal fluid would necessitate a quantitative estimation of choline in this fluid under various conditions, a proceeding which appears to present very considerable difficulties.

In my experiments I have employed seven dogs and one cat. In six dogs and in the cat both sciatic nerves were divided and a long piece resected; the blood was taken a variable number of days after the operation. The results are put together in the following table:—

17. *Loc. cit.*
18. *Loc. cit.*
19. *Loc. cit.*
20. *Journ. of Physiol.*, Vol. XXXIII, p. 220, 1905-6.

No. of Experiment	No. of days since resection of nerves in the animal from which the pathological blood in fifth column was taken	Weight of animal used for blood pressure experiment	Effect of injections of extract of 110 c.c. of normal dog's blood on the blood pressure before Atropine	Effect of injections of extract of 110 c.c. of pathological blood on the blood pressure before Atropine	Normal extract as in third column after Atropine	Pathological extract as in fourth column after Atropine
1	5	6·5 k.	Very slight temporary rise as often seen with saline injection	Very slight temporary fall 0·2 cm.	Exactly same result as before Atropine	Exactly same result as before Atropine
2	7	6·5 k.	Slight rise	No effect on B.P.	,,	,,
3	9	4·2 k.	Slight fall	Slight fall 0·5 cm.	,,	,,
4	11	4·3 k.	,,	Slight fall 0·6 cm.	,,	,,
5	12	5·3 k.	Slight fall 0·3 cm.	Slight fall 0·3 cm.	,,	,,
6	17	5·2 k.	Slight fall 0·7 cm.	Slight fall 0·6 cm.	,,	,,

It may be mentioned that because dogs were employed instead of cats in these experiments the precaution was taken to compensate for their larger size by injecting an extract from a large quantity of blood four to five times as much as the quantity employed by Halliburton.[22]

In order to produce even more extensive degeneration, in one dog a portion of the dorsal spinal cord about an inch in length was excised. After eight days the animal was killed, and its blood subjected to the physiological test. The effect produced upon the blood pressure was identical with that produced by an extract of an equal quantity of normal blood.

It will be seen that there is no progressive increase in the depressor effect such as was described by Halliburton. In fact, in no case did the extract of pathological blood give a fall greater than that obtained by the extract from a normal animal. Further, the effects were precisely the same after the administration of atropine as before.

This result completely confirms the observations of Vincent and Cramer, who arrived at the conclusion that the substance present in the pathological blood which gave a fall of blood pressure in Halliburton's experiments was not choline, nor indeed anything arising from morbid processes, but some substance common to normal blood and to all animal tissues.

Chemical

I have applied the chemical test as recommended by Rosenheim to the blood of animals operated upon as described above. Using a pure choline solution, the formation of dark brown crystals can be easily verified. They are very few in number, and small when a solution of choline of 1-200000 is used. I have, however, been unable to find any similar crystals in the blood of any of the animals experimented upon. An abundant crop of the platinum-chloride crystals can be obtained from both normal and pathological blood if the old test originally recommended by Halliburton is used. This, again, is confirmatory of the result obtained by Vincent and Cramer, and renders very doubtful the present claim of Halliburton that 'the obtaining of a large crop of crystals, whether they be those of the choline salt or a mixture of the potassium and choline salts, is diagnostic of an extensive breakdown in nervous tissues.'[23]

22. Such a precaution is really not necessary, because, together with the increase in size, there is an increase in the weight of the sciatic nerves, and, consequently, of the amount of nervous tissue degenerating after section of the nerve.

23. 'Oliver Sharpey Lectures,' *Brit. Med. Journ.*, May 4th, 1907.

The addition of iodine solution to these crystals does not cause the formation of choline periodide. Some brown crystals are formed, irregular in shape and varying in size, but none are oblong—the characteristic shape of the periodide crystals—neither do any of them resolve themselves into 'oily droplets' on drying of the solution, but retain their form days or weeks after drying has occurred.

I have been greatly disappointed to find that none of the statements which constitute the basis of the choline hypothesis can be verified. The observations upon which the hypothesis rests are the outcome of investigations which have not been sufficiently controlled by the examination of normal tissues and fluids, since all the effects alleged to be characteristic of pathological conditions can be obtained in the normal state.

That my results are not due to faulty technique can be proved by the following calculation :—

Taking the total weight of the central nervous system as 1,400 grammes, it can be calculated on the basis of our present knowledge of the chemistry of nervous tissue that about 8·5 grammes of choline are contained therein. This follows from the observations of Coriat,[24] who found that 10 grammes of moist brain tissue yield 0·547 gramme of choline, as against the theoretical amount of 0·0584 gramme of choline calculated from Koch's figures. It follows then that *1 gramme of moist brain substance contains 0·006 gramme of choline.* The average volume of total blood is 4,700 c.c. After a sudden destruction of 1 gramme of brain substance the blood of a patient would contain 0·006 gramme of choline. This corresponds to a dilution of 1 in 800,000, therefore 20 c.c., the volume recommended and generally employed by Mott and Halliburton, would contain 0·000025 gramme of choline = one-fortieth of a milligramme. This only holds good if 1 gramme of brain is destroyed suddenly, as choline is oxidised in the organism. As a rule, degenerative processes destroying 1 gramme of brain substance would be spread over a considerable time, so that the amount of choline at any particular moment would be much smaller than is given above.

Now, none of the tests hitherto employed are sufficiently delicate to indicate choline in such degrees of dilution.[25] Any statement hitherto made to the effect that choline has been demonstrated in the blood may therefore be taken as showing either that the test by which it has been

24. *American Journ. of Physiol.*, Vol. XII, p. 353, 1905.

25. Some very delicate tests have been devised by Gumprecht and by Reid Hunt, but these tests have as yet not been applied to the investigation of this question.

recognised is fallacious, or if that can be excluded, that the choline which *is* present is not derived from the degeneration of nervous tissues.

It is interesting to compare these figures with the amount of choline found in the blood by Halliburton and by Donath. The former observer found in the blood of cats he experimented upon choline up to the amount of 0·0052 to 0·0078 per cent. at a time when the degeneration was stated by him to be at its height. Taking the volume of the blood of a cat at 150 c.c., these figures would correspond to a sudden destruction of more than 1 gramme of nervous tissue at the time when the animal, a cat, was killed. As choline is stated to be abundant from the eighth to the thirteenth day after section of the nerves, the amount of nervous tissue destroyed in order to account for the amount of choline alleged to be present would be enormous.

With regard to the cerebro-spinal fluid, the physiological effect on the blood pressure produced by injections of 10 c.c. of cerebro-spinal fluid from cases of general paralysis is compared with the effect of injections of 1 to 5 c.c. of a 0·2 per cent. solution of pure choline. The amount of choline injected in the latter case is 0·002 to 0·01 gramme, and is, according to Halliburton,[26] comparable to the amount of the base presumably present in pathological cerebro-spinal fluid. We have seen that (if calculated for the total cerebro-spinal fluid = 100 c.c.) such an amount could be produced only by the *sudden* destruction of about 3 to 15 grammes of nervous tissue.

These considerations completely justify the statement with which Vincent and Cramer concluded their paper, and to which Halliburton has taken exception. Indeed, it appears, *a priori*, improbable that the substance in these pathological specimens of blood should be choline derived from nervous tissue. That such comparatively slight destruction of nervous elements as takes place even in extensive disease should supply sufficient choline to the blood to give the physiological test seems scarcely conceivable, especially when we remember that choline is not a very powerful depressor substance.

Donath[27] determined the amount of choline in the cerebro-spinal fluid of patients. Taking the volume of this fluid at the minimal value of 100 c.c., his results indicate the presence of from 20 to 40 milligrammes of choline. This would correspond to a sudden destruction of 3 to 7 grammes of nerve tissue, and that in cases of epilepsy, both Jacksonian and of idiopathic, and of neurasthenia.

26. *Croonian Lectures*, p. 51, 1901.

27. *Deutsche Zeitschrift für Nervenheilkunde*, Vol. XXVII, p. 71, 1904.

Quite recently Halliburton, although admitting that the platino-chloride crystals obtained from blood are due partly to the presence of potassium, makes the claim[28] that the obtaining of a large crop of crystals, 'whether they be of the choline salt or a mixture of the potassium and choline salts, is diagnostic of extensive breakdown in nervous tissues. The contrast between such cases and the insignificant yield from normal blood is most striking. This is quite intelligible, when we take into account the very high percentage of potassium that nervous tissues contain.' If the presence of potassium in the blood leads to the formation of these crystals, it may be asked why the considerable amount of potassium which is known to be present in normal serum should give an insignificant yield of crystals, while an increase which must necessarily be very limited so long as the kidneys are performing their work, should produce an abundant crop. As a matter of fact, I have never been able to find such a difference, having obtained as abundant a crop of crystals from normal blood as from the blood of animals with recent nerve lesions. The idea that degenerative processes in the nervous system should lead to such an increase in the amount of potassium in the blood that it can be recognised qualitatively by the yield of the platino chloride crystals is, as in the case of the choline hypothesis, based on a complete misconception of the quantitative relationships. Taking the minimal values given[29] for the amount of potassium in the central nervous tissues as 1·7 per mille, it follows that 1 gramme of brain contains 0·0017 gramme K_2O; taking the minimal value of K_2O content of blood as 0·2 per mille and the total volume again as 4,700 c.c., we find that in man a sudden destruction of 1 gramme of nervous tissue would add 0·0017 gramme K_2O to 0·9 gramme K_2O in the total blood. This is a difference smaller than that which appears in Abderhalden's[30] very careful analyses of samples of blood from two different animals of the same species. In 20 c.c. of blood the amount of potassium would be increased by 0·000007 gramme, or less than 1/100th of a milligramme.

In smaller animals, e.g., in cats, the proportion between the amount of potassium normally present in the blood and the amount which may be set free at any given moment by degenerative processes in nervous tissue remains of course essentially the same, since with a diminished volume of blood we have also a diminished amount of nervous tissue.

28. 'Oliver Sharpey Lectures,' *Brit. Med. Journ.*, May 5th, 1907.

29. See, for instance, Hammarsten, *Text Book of Physiological Chemistry*, p. 414, 1904.

30. *Zeitschrift. f. physiolog. Chem.*, XXV, p. 65, 1898.

It is clear that even the most extensive degeneration which can possibly occur is quite incapable of yielding sufficient choline and potassium to account for the striking contrast in the yield of crystals to which Halliburton refers. If the preliminary statement by the same author is correct, that actual estimations of the potassium content of the blood show an increase in some cases of acute degenerative diseases, it is equally clear that this increase must be due to other factors than the liberation of potassium by the degenerating nervous tissue.

The smaller volume of cerebro-spinal fluid makes this a more likely place wherein to find the products of degeneration of nervous tissue. But from the data given above it follows that the destruction of nervous tissue would have to be very considerable, 1 gramme at least, before one would be able to detect choline in the cerebro-spinal fluid by means of Rosenheim's modification of the iodine test, which is stated to indicate choline in a dilution of 1 : 20000. Kaufmann,[31] who collected a litre of cerebro-spinal fluid from various cases of nervous diseases and who was thus in a position to investigate the subject by exact chemical methods instead of relying upon micro-chemical tests, was unable to isolate choline from the cerebro-spinal fluid. The fact that Kaufmann found another organic base to be present which, although having many reactions in common with choline, proved on more detailed chemical examination to be altogether different from choline, detracts from the value of observations based upon micro-chemical reactions for the detection of a substance which, if it is present at all, is present only in fractions of a milligramme.

Those who first advanced the choline hypothesis were under the impression that the amount of choline liberated in diseases could be measured by multiples of milligrammes; and the relatively great quantities of choline liberated, which their experiments appeared to indicate, was one of the strongest arguments in favour of their hypothesis. We know now that the amounts which can possibly appear as the result of degenerative processes in nervous tissue are exceedingly minute, and the question arises whether such small amounts may not be derived from other tissues. Lecithin is a constituent common to all cells. A destruction of a great number of white or red blood corpuscles may just as well lead to choline being set free. Thus the leucocytosis occurring in the cerebro-spinal fluid in some diseases, or the degeneration of a mass of red blood corpuscles after a haemorrhage may be a source of choline.

31. *Neurologisches Centralblatt*, Vol. XXVII, p. 260, 1908.

Summary

1. No choline can be detected in normal blood, provided that the decomposition of the lecithin be prevented in the methods employed. Nor can choline be detected in blood of animals after extensive lesions of the central or peripheral nervous system.

2. The maximal quantity of choline which would be set free in processes of degeneration is too small to be detected in the blood by any of the methods hitherto employed. The same holds good for potassium.

3. The physiological and chemical tests given by pathological blood, and alleged to be characteristic of choline, are exhibited in exactly the same manner and to the same degree by normal blood.

4. The micro-chemical reactions recommended for the detection of choline are given irregularly both by normal and pathological cerebro-spinal fluid. The presence in the cerebro-spinal fluid of a substance giving the micro-chemical reactions of choline cannot, therefore, be considered as indicative of degenerative changes in the nervous system; and in view of Kaufmann's results it may even be doubted whether any of the micro-chemical tests at present in use are specific for choline. Whether such changes increase the amount of choline in the cerebro-spinal fluid by fractions of a milligramme, and whether the same effect may not be produced by the disintegration of tissue other than nervous, will have to be determined by further experimental investigation.

THE BIURET REACTION AND THE COLD NITRIC ACID TEST IN THE RECOGNITION OF PROTEIN

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In all books on physiological chemistry and clinical diagnosis one is recommended to place great reliance on the biuret reaction. In the course of some investigations on the comparative reliability of various tests for serum albumen I found that I obtained very contradictory results with this test, and therefore I think it would be of interest to give the results which I obtained in an exhaustive series of tests.

The usual method given in books for carrying out this test is: that one adds to the albumen solution some soda solution and then, drop by drop, a very dilute solution of copper sulphate, a blue precipitate appearing which, on shaking, dissolves with a pink tinge, finally changing to a reddish violet.[1] But on doing this I found that one could not be certain of obtaining the reaction in a urine which by other tests, such as cold nitric acid, acetic acid and heat, and potassium ferrocyanide and acetic acid, gave clear indications of the presence of albumen.

It was therefore necessary to make up a solution containing a definite quantity of serum albumen and to repeat the tests. On doing this I found that it was impossible to make certain of obtaining constant results unless one used solutions of soda and copper sulphate of definite strength, for the addition of the soda solution in varying amounts made a great difference in the result of the test.

It is generally recognised that it is necessary to add a dilute solution of copper sulphate, and I found that, although with a high percentage of albumen a $2\frac{1}{2}$ per cent. solution of copper sulphate may be used, yet for all practical purposes the solution of copper sulphate should not be stronger than 1 per cent.

Another point on which the various text-books, which I have had an opportunity of consulting, lay little stress is on that of heating, and

1. Some text books advise adding the copper sulphate solution before the soda solution.

although one can obtain a more or less definite result in the cold, I found that the reaction is much more marked on heating. In fact, in a very weak solution of albumen the violet is not perceptible until the solution is boiled.

In consequence of these difficulties I decided to work with solutions of albumen, soda and copper sulphate of definite strength, and the original solutions which I made up were as follows:—

I. Albumen solution. Distilled water containing 0·2 per cent. of albumen.

II. A solution containing sodium hydrate 10 grammes and distilled water to 100 c.c.

III. A solution containing re-crystallised copper sulphate 5 grammes and distilled water to 100 c.c.

Solution I.—Beginning with the original solution of 0·2 per cent. of albumen, I made an exhaustive series of dilutions with distilled water, these diluted solutions varying in strength from 0·05 per cent. to 0·00033 per cent. of albumen. A number of these dilutions are referred to in Tables I and II.

Solution II.—As a result of many tests in which I used varying percentages of sodium hydrate, in albumen solutions of different strengths, I found that a 10 per cent. solution of sodium hydrate gave the most constant results. The quantity of this soda solution used in each test—final result—is given in Tables I and II.

Solution III.—The 5 per cent. solution of copper sulphate was too strong, for even in the original solution of albumen (Solution I) a brown coloration with a white flocculent precipitate was obtained on the addition of one drop of this copper solution with soda. I therefore diluted to a $2\frac{1}{2}$ per cent. solution, but, after three or four estimations, I found this in turn too strong, and it was necessary to dilute to a 1 per cent. solution.

Table I shows results on using a $2\frac{1}{2}$ per cent. solution.
Table II ,, ,, 1 ,, ,,

For adding the copper sulphate and soda solutions drop by drop, I used an ordinary glass pipette, that is, ordinary glass tubing drawn to a small calibre at one end.

It was necessary to do a great many tests before being able to determine the best method of performing the test. I found as follows:—

(*a*) 10 c.c. of the albumen solution is the best amount to use.

(*b*) In all the albumen solutions of from 0·004 per cent. to the limit

of delicacy one drop of copper sulphate (1 per cent.) and five drops of sodium hydrate (10 per cent.) gave the best results.

In the albumen solutions stronger than 0·004 per cent. two to ten drops of copper sulphate (1 per cent.) can be added, according to the strength of the solution of albumen.

In the solutions from 0·05 per cent. to 0·012 per cent. of albumen I used 2½ per cent. copper sulphate solution. (See Tables I and II.)

(*c*) The copper sulphate should be added first, then the soda solution. The contents of the test tube should then be mixed by inverting twice.

(*d*) After adding the copper and soda the solution should be heated to boiling, when the violet is intensified.

All text-books I have consulted mention that the colour obtained in the biuret reaction is pink or red, changing to violet. I was unable to obtain this play of colours, as all my positive tests gave the violet colour at once. I found that the stronger the solution of albumen the more marked was the violet colour—gradually diminishing with dilution—and, also, that in strong solutions of albumen the violet is intensified by the addition of copper sulphate solution drop by drop, boiling between drops.

In the very dilute solutions of albumen there is some difficulty in recognising the violet colour. I found that the colour was best seen as follows:—

1. In the meniscus.
2. On looking down the test tube—against white.
3. On comparing the albumen solution with a test tube containing an equal quantity of distilled water. The two tubes should be held side by side and compared:—

(*a*) On looking down both—against white.
(*b*) In the meniscus—

The best light is that which strikes from above. Both tubes should be slanted towards the operator at about 35°. One should hold the tubes above the head and look up through the meniscus from below, against a dark background.

TABLE I

Albumen per cent.	$CuSO_4$ Solution 2½ per cent.	NaOH Solution 10 per cent.	Result	Remarks
0·050	3 drops	5 drops	Violet	Reaction well marked
0·025	3 ,,	5 ,,	,,	,, ,, ,,
0·016	2 ,,	5 ,,	,,	Reaction marked
0·012	1 drop	5 ,,	,,	,, ,,

In the next dilution—solution of 0·008 per cent. of albumen—one drop of copper sulphate solution ($2\frac{1}{2}$ per cent.) and five drops of sodium hydrate solution (10 per cent.) were added, and the solution at once turned brown. On boiling, the brown colour deepened with the formation of a white flocculent precipitate. The result might be shown thus:—

Albumen per cent.	$CuSO_4$ Solution $2\frac{1}{2}$ per cent.	NaOH Solution 10 per cent.	Result	Remarks
0·008	1 drop	5 drops	No violet	Solution at once turns brown

In all the succeeding tests I used a 1 per cent. solution of copper sulphate.

TABLE II

Albumen per cent.	$CuSO_4$ Solution 1 per cent.	NaOH Solution 10 per cent.	Result	Remarks
0·0080	2 drops	5 drops	Violet	Colour deepened on addition of one more drop $CuSO_4$ solution.
0·0040	1 drop	,,	,,	
0·0026	,,	,,	,,	
0·0020	,,	,,	,,	
0·0016	,,	,,	,,	
0·0013	,,	,,	,,	
0·0011	,,	,,	,,	
0·0010	,,	,,	,,	
0·00088	,,	,,	,,	Violet faint before boiling.
0·00080	,,	,,	,,	
0·00072	,,	,,	,,	
0·00066	,,	,,	,,	Violet very faint before boiling.
0·00061	,,	,,	,,	
0·00056	,,	,,	,,	Using distilled water as comparison, violet is just perceptible before boiling.
0·00053	,,	,,	,,	
0·00050	,,	,,	,,	
0·00047	,,	,,	,,	Violet imperceptible before boiling except on comparison with distilled water.
0·00044	,,	,,	,,	
0·00042	,,	,,	,,	
0·00040	,,	,,	,,	Violet imperceptible before boiling even when compared with distilled water, and just perceptible after boiling when compared with distilled water.
0·00038	,,	,,	,,	On standing for two minutes solution turns brown.
0·00036	,,	,,	,,	On standing for one minute solution turns brown.
0·00034	,,	,,	,,	On standing for twenty seconds solution turns brown.
0·00033	,,	,,	No violet	Solution at once turns brown, with the formation of a white flocculent precipitate.

From the results which I obtained in a watery solution of albumen, I decided to make further tests, using as my original solution urine and albumen. I therefore took a urine free from albumen and added albumen to make a 2 per cent. solution.

As in the previous tests, I made a great many dilutions with distilled water, and carried out investigations as before. The more or less abridged results are seen in Table III.

In these reactions I used sodium hydrate (10 per cent.) and copper sulphate (1 per cent.).

In the albumen solutions stronger than 0·04 per cent. more than one drop of copper sulphate solution was used, but in 0·04 per cent. and all the succeeding solutions to the limit of delicacy I found that one drop of copper sulphate solution gave the best results. In all cases five drops of sodium hydrate gave most constant results.

On account of the added colour of the urine, I found it more difficult to recognise the violet colour in this than in the first series of tests.

TABLE III

Albumen per cent.	$CuSO_4$ Solution 1 per cent.	NaOH Solution 10 per cent.	Result	Remarks
2·0	Five drops	Five drops	Violet	Colour deepened to maximum on addition of ten more drops of $CuSO_4$—drop by drop—boiling between drops.
0·20	,,	,,	,,	Colour deepened to maximum on addition of five more drops of $CuSO_4$—drop by drop—boiling between drops.
0·040	One drop	Five drops	Violet	Reaction good.
0·020	,,	,,	,,	
0·014	,,	,,	,,	
0·010	,,	,,	,,	
0·0066	,,	,,	,,	
0·0050	,,	,,	,,	
0·0040	,,	,,	,,	
0·0033	,,	,,	,,	Violet faint before boiling.
0·0028	,,	,,	,,	
0·0025	,,	,,	,,	
0·0022	,,	,,	,,	Violet very faint before boiling.
0·0020	,,	,,	,,	
0·0016	,,	,,	,,	
0·0014	,,	,,	,,	Violet perceptible before boiling only on comparison with distilled water.
0·0012	,,	,,	,,	
0·0011	,,	,,	,,	
0·0010	,,	,,	,,	Violet imperceptible before boiling. After boiling violet just perceptible on comparison with distilled water.
0·0009	,,	,,	,,	But changing almost at once to brown, with the formation of a white flocculent precipitate.

Having determined the delicacy of the test in a watery solution of albumen, and in a urine containing albumen diluted with water, I next tried to obtain the limit of delicacy in a urine containing albumen when urine free from albumen was used for dilution.

According to Hammarsten,[1] an excess of creatinin reduces copper sulphate, and Kellas and Wethered[2] have shown that an excess of uric acid and urates, as well as creatinin, causes a reduction of copper sulphate.

The first urine which I used for diluting contained an excess of uric acid, and in these solutions I was unable to obtain the violet colour except in the stronger solutions of albumen—0·2 per cent. and stronger.

I then did further tests with solutions made by diluting the albuminous urine with urines containing an excess of urates and creatinin, but was unable to obtain positive results except in the stronger solutions of albumen—0·2 per cent. and stronger.

While working on the biuret reaction it occurred to me that it would be of interest to determine the delicacy of the cold nitric acid test for albumen, as this test is so well known and so commonly used.

The limit of delicacy of this test given in text-books is 0·002 per cent., but I was able to obtain positive results in very much weaker solutions, as will be seen in Table IV.

To obtain satisfactory results in doing the test the following points should be noted:—

(*a*) 12 c.c. of albumen solution should be used.

(*b*) 2 c.c. of nitric acid should be used.

(*c*) A pipette should be used for adding the nitric acid to the albumen solution. This pipette is made as follows: One end of an ordinary piece of glass tubing, 5 mm. diameter, is drawn to a calibre which allows the nitric acid to escape from the pipette by the drop and not in a continuous stream, when the finger is removed from the large end of the pipette.

The pipette should be not less than 28 cm. in length.

Technique of the Test.—Take 12 c.c. of albumen solution in a test tube. Draw up 2 c.c. of nitric acid in the pipette, place the finger over the end and carefully lower the pipette into the albumen solution until the point of the pipette is on the bottom of the test tube. Remove the finger from the pipette and allow the nitric acid to run in. When no more nitric acid runs in place the finger over the end of the pipette and carefully withdraw it. Care must be taken not to shake the test tube during the test.

By this method the line of demarcation between the albumen solution and the nitric acid is narrow and very distinct, thus facilitating the recognition of the albumen ring as it forms.

1. *Physiologischen Chemie.*
2. *The Lancet*, October, 1906.

My first series of tests was done with diluted solutions of my original albumen solution, that is, distilled water containing 0·2 per cent. of albumen. The results in a number of these solutions are given in Table IV.

One finds in text-books the statement that, for the cold nitric acid test for albumen to be positive, the albumen ring should occur within five minutes, but on referring to Table IV it will be seen that in very dilute solutions of albumen the ring did not occur until as long as fifteen minutes, and reached its maximum density in one hour after adding the nitric acid.

TABLE IV

Albumen per cent.	Nitric Acid	Result	Time		
0·20	2 c.c.	Positive	In 20 seconds		
0·0020	,,	,,	,, 30 ,,		
0·0010	,,	,,	,, 1 minute		
0·00066	,,	,,	,, 1 ,,		
0·00050	,,	,,	,, 1½ minutes		
0·00040	,,	,,	,, 2 ,,		
0·00033	,,	,,	,, 2 ,,		
0·00027	,,	,,	,, 2½ ,,		
0·00025	,,	,,	,, 3 ,,		
0·00022	,,	,,	,, 3¼ ,,		
0·00020	,,	,,	,, 3½ ,,		
0·00018	,,	,,	,, 4 ,,		
0·00016	,,	,,	,, 4 ,,		
0·00015	,,	,,	,, 4½ ,,		
0·00014	,,	,,	,, 5 ,,		
0·00013	,,	,,	,, 5 ,,		
0·00012	,,	,,	,, 5½ ,,		
0·000117	,,	,,	,, 6 ,,		
0·000111	,,	,,	,, 6½ ,,		
0·000105	,,	,,	,, 7 ,,		
0·000100	,,	,,	,, 7½ ,,		
0·000095	,,	,,	,, 8 ,,		
0·000090	,,	,,	,, 9 ,,	Ring most marked in 15 minutes	
0·000086	,,	,,	,, 10 ,,	,, ,, 20 ,,	
0·000083	,,	,,	,, 11 ,,	,, ,, 25 ,,	
0·000080	,,	,,	,, 12 ,,	,, ,, 30 ,,	
0·000076	,,	,,	,, 13 ,,	,, ,, 35 ,,	
0·000074	,,	,,	,, 14 ,,	,, ,, 40 ,,	
0·000071	,,	,,	,, 14 ,,	,, ,, 45 ,,	Faint
0·000068	,,	,,	,, 15 ,,	,, ,, 50 ,,	
0·000063	,,	,,	,, 15 ,,	,, ,, 1 hour	
0·000060	,,	,,	,, 15 ,,	,, ,, 1 hour.	Very faint

For my next series of tests I used urine containing 2 per cent. of albumen—made by taking a urine free from albumen and adding albumen to make 2 per cent. Taking this as my original solution I made a large number of dilutions, using for dilution a urine free from albumen.

The results in a number of these solutions are given in Table V.

In doing the cold nitric acid test in albuminous urine containing an excess of uric acid, after adding the nitric acid a cloudiness appears in a few minutes, gradually increasing in density all through the urine from the top of the solution down to the nitric acid ring. To differentiate between this cloudiness, due to uric acid, and the albumen ring, the urine should be heated gently just above the nitric acid ring. The cloudiness due to uric acid disappears and the albumen ring remains. There will then be seen in such cases, in order from below, the nitric acid ring, the white albumen ring, a clear space, and above this more or less cloudiness.

It must be remembered in this connection that albumose may be present also. If so, on gently heating just above the nitric acid ring, the depth of the white precipitate will diminish, since the albumose is dissolved and the albumen is left. By this means one can obtain a rough idea as to the quantity of albumose present.

In Table V it will be seen that in very weak solutions of albumen the albumen ring did not appear until ten minutes after the addition of the nitric acid.

TABLE V

Albumen per cent.	Nitric Acid	Result	Time			
0·04	2 c.c.	Positive	In	1	minute	
0·0040	,,	,,	,,	1½	minutes	
0·0020	,,	,,	,,	2	,,	
0·0013	,,	,,	,,	2½	,,	
0·0010	,,	,,	,,	3	,,	
0·00080	,,	,,	,,	3½	,,	
0·00060	,,	,,	,,	4	,,	
0·00057	,,	,,	,,	4½	,,	
0·00050	,,	,,	,,	5	,,	
0·00044	,,	,,	,,	5½	,,	
0·00040	,,	,,	,,	6	,,	
0·00036	,,	,,	,,	6½	,,	
0·00033	,,	,,	,,	7	,,	
0·00030	,,	,,	,,	7	,,	
0·00028	,,	,,	,,	8	,,	
0·00026	,,	,,	,,	8½	,,	
0·00025	,,	,,	,,	9	,,	
0·00023	,,	,,	,,	9½	,,	
0·00022	,,	,,	,,	9½	,,	Faint
0·00021	,,	,,	,,	10	,,	
0·00020	,,	,,	,,	10	,,	Very faint

CONCLUSIONS

A. *Biuret Reaction.—Limit of Delicacy.*

I. In a watery solution of albumen is 0·0004 per cent. or four parts of albumen in 1,000,000 parts of distilled water.

II. In albuminous urine diluted with distilled water is 0·001 per cent. or one part of albumen in 100,000 parts of urine and distilled water.

III. In albuminous urine diluted with urine free from albumen.

The biuret reaction is very difficult to obtain in concentrated urines containing an excess of uric acid, urates or creatinin. In such cases it is only in the stronger solutions 0·2 per cent.—that the reaction is good. In the weaker solutions of albumen the violet colour may be obtained, but almost at once the colour changes to brown.

In most cases no violet colour is obtained, the solution at once turning brown with the formation of a white flocculent precipitate.

B. *Cold Nitric Acid Test.—Limit of Delicacy.*

I. In a watery solution of albumen is 0·00006 per cent. or six parts of albumen in 10,000,000 parts of distilled water.

II. In albuminous urine diluted with urine free from albumen is 0·0002 per cent. or two parts of albumen in 1,000,000 parts of urine.

My best thanks are due to Professor Harley and Dr. Goodbody for much kind assistance and advice.

THE PROPERTIES AND CLASSIFICATION OF THE OXIDIZING ENZYMES, AND ANALOGIES BETWEEN ENZYMIC ACTIVITY AND THE EFFECTS OF IMMUNE BODIES AND COMPLEMENTS

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The presence in plant and animal juices of bodies possessing the properties of ferments which act as oxidizing agents for unstable bodies, such as the guaiaconic acid of guaiacum resin, was first demonstrated by Schönbein[1] in 1856.

The action of these bodies was studied in greater detail by Bertrand[2] in a series of papers beginning in 1894. Bertrand first showed that the formation of Japanese lacquer was due to the oxidation of a substance present in the plant juice of certain species of *Rhus* by a ferment which he termed *laccase*. The browning and blackening of the cut surfaces of fruits and other parts of vegetable tissues is also due to the action of oxidizing ferments.

From the juice of the potato an oxidizing ferment termed *tyrosinase* was obtained, which acted vigorously upon the isolated tyrosin, yielding a dark brown substance, richer in oxygen than the tyrosin from which it was formed.

These browning or blackening ferments are distinct in nature from the oxidizing ferments present in most fresh plant juices, and also in pus, milk, blood, and extracts of some animal organs, which possess the property of turning tincture of guaiacum blue, of oxidizing and thereby rendering coloured certain phenolic bodies, or of setting free iodine from a solution of hydriodic acid.

Bertrand showed that this latter class of oxidizing ferments was present in many different types of plant, and he expressed the view that they were universally present in all plants.

It is, however, certain that they are not present in all plant juices, although they may be present in other parts of the same plant. Our own experiments recorded below show, for example, that the class of oxidizing

1. *Zeitsch. f. Biol.*, Bd. III, S. 325.
2. *Compt. rend.*, p. 1215, 1894.

ferments is absent in the fresh juice of the fruit of the lime, lemon, and orange, but present in the crushed seeds of lemon and orange. Other experiments have demonstrated to us that their distribution in different parts of a root or fruit varies widely, and that they are present in greatest quantity in the region containing the greatest abundance of respiratory vessels.

Thus a section across a carrot stains almost at once in the protoxylem, and the staining radiates out from this along the path of vessels, while internally to the protoxylem there is very little staining (see Expt. XIX).

It was found by many observers that the guaiacum test for these oxidizing ferments often failed, especially in fluids of animal origin and when freshly made tincture of guaiacum was used for testing.

Further, it was found that in such cases the blueing could still be obtained, provided either hydrogen peroxide were added or some organic form of peroxide, containing oxygen linked in the peroxide form.

A similar oxidation is seen in the well-known test for traces of blood in urine or other fluids in which tincture of guaiacum and ozonic ether (a form of peroxide) are added to the suspected fluid. This test also demonstrates that bodies other than oxidizing ferments occurring in living tissues are capable of giving the guaiacum blueing. Many inorganic and organic bodies also give it, such as sulphurous and nitrous acids, ferrous salts, and potassium permanganate.

The substances causing this reaction may be either oxidizing or reducing bodies, so long as they can act as oxygen carriers.

In this connection it may also be pointed out, as has been done by Bach,[3] that all the oxidations shown to occur with this class of oxidizing enzymes, viz., oxidation of hydriodic acid, of the aromatic amines and phenols, and of the guaiaconic acid of the guaiacum resin, possess the common factor of a moveable or *dynamic* hydrogen atom in the molecule. Between this hydrogen atom and the atom of the oxygen in the peroxide form there is already a strong tendency to reaction, and the catalyst simply increases this tendency.

On account of the too great readiness with which the guaiacum underwent the oxidation change giving the characteristic blueing, and also on account of the fact that blood, and other oxidizing bodies not ferments, readily yielded the guaiacum test, other more definite tests were sought out. A considerable number of tests have been described in which phenols or amido-phenols are oxidized to bodies which possess various

3. *Berichte d. deut. Chem. Gesellsch.*, Vol. XL, p. 230, 1907.

colours. These colour tests for oxidizing enzymes are all very similar, and when one of them has been obtained, or not obtained, in any given case, a similar result is usually obtained with all the others, probably because the potential of chemical energy required for oxidation lies at about the same level for all of them.[4]

The results of carrying out a number of these colour tests will be given later, but one important difference which we have found may be stated here in general terms, since, in our opinion, it leads to certain important conclusions regarding the classification and mode of action of this class of oxidizing enzymes different from those generally accepted at the present time. This result is that none of the colour tests, with the exception of the guaiacum test, are appreciably increased in velocity, or catalysed, by the solution of the oxidizing ferment alone, except when the fresh juice is taken immediately after its preparation (see Expt. VII), and are only so catalysed when hydrogen peroxide or some other form of peroxide is added at the same time.

The reasons why the guaiacum test is often positive when the others are negative are (1) the presence of organic peroxide in the guaiacum itself, and (2) that the oxidation occurs much more readily than with the other colour tests with the oxy- and amido-phenols, and such like bodies, and so the naturally occurring organic peroxide of the juice or of the reagent is sufficient to give an oxidation. Whereas the more stable amino-acids only break up with appreciable velocity in presence of the more readily decomposed, and hence more powerful, free hydrogen peroxide, which has been artifically added.

No attempt will be made in this paper to give a full account of the very wealthy literature of the oxidizing ferments,[5] since accounts in English have recently appeared in 'The Nature of Enzyme Action,' by Bayliss,[6] and the 'Intracellular Ferments' of Vernon;[7] but some outline of the classification introduced by Bach and Chodat, and of the basis for that classification, must be given in order that our own views and experiments may be more easily followed. It may be added that this classification has been almost unanimously adopted by subsequent writers.

4. Minor differences in reactivity of the different colour reagents are given in the experimental part of the paper.

5. Citations of the literature are also to be found in the comprehensive review by Bach and Chodat, *Biochemischen Centralblatt*, 1903; in 'Über tierische Peroxydasen,' Ernst von Czyhlarz u. Otto von Fürth, *Beitrage z. Chem. Physiol. u. Path., Zeitsch. f. Biochemie*, Bd. X, S. 358, 1907; Bach, *Berichte*, 1904 to date; Spence, this Journal, Vol. III, p. 165, 1908.

6. *Monographs on Bio-Chemistry.* Edited by Aders-Plimmer and Hopkins. Longmans, Green and Co., 1908.

7. Published by John Murray for Physiological Laboratory, University of London.

The experimental work of Bach and Chodat, especially that of a quantitative nature on the action of these oxidizing ferments, is, in our opinion, sound, and forms an excellent basis for further work; but we cannot agree that their experimental observations give sufficient ground for the classification they have adopted nor for belief in two classes of oxidizing ferments, the *oxygenases* and the *peroxidases*, which they postulate, forming together a mixture of ferments corresponding to the old *oxidases*.

In our opinion there is but one class of such enzymes concerned, which, since they act only in presence of oxygen linked as a peroxide, might still be known as the peroxidases. The oxygenases do not exist as ferments at all; there is no use for such a term, and it might be allowed to drop out. The oxygenases are simply preformed peroxides in juice or reagent, and not in any sense ferments.

The experimental basis, and as far as we can discover the only one, for a belief in the oxygenases is that certain juices, such as that of the potato, give at once a blue colour with fresh guaiacum alone without added hydrogen peroxide, while other fresh juices, such as those of radish and cucumber,[8] give absolutely no blue coloration with the fresh guaiacum tincture until hydrogen peroxide, or some other peroxide, is added, when almost at once a strong blue is obtained.

Further, if the potato juice be heated for some hours to about 60° C. it in most cases loses the power of blueing spontaneously, and now only gives the blue colour when a peroxide is added.[9]

The explanation of this is easy on Bach and Chodat's classification, which represents that there are two enzymes present in the juices, viz., *oxygenase* which manufactures peroxides from the material at hand in the juice, and a *peroxidase* which then activates this peroxide and causes it to attack the oxidizable body yielding the colour test and oxidize it, so producing the colour.

We submit that there is no proof that there is an enzyme or enzymes forming the class of the oxygenases and producing organic peroxides, and that the whole difference between the two classes of juices is simply that

8. Bach and Chodat, *loc. cit.*

9. This destruction of organic peroxides (or of oxygenases according to Bach and Chodat's views) is not quite so easy as it is usually described, for potato juice may be briskly boiled in a test-tube for half a minute without destroying completely all the store of peroxide. If it is again boiled, however, or if it be thoroughly boiled for some minutes in a beaker so that all parts are boiled, the peroxide is completely destroyed. This refractiveness was discovered by Woods (*Bull. U.S. Dept. of Agriculture*, No. 18, p. 17) in the case of the tobacco oxidase, and by him attributed to part of the oxidase existing as a zymogen. There is little doubt, however, that the result is due to incomplete destruction of the peroxide and peroxidase.

one has a store of peroxides and the other has not, and, further, that these peroxides are thermally unstable and are destroyed by heat.

After we have put forward the evidence in favour of this view, we shall draw attention to the similarity between this simpler scheme for the activity of the oxidizing ferments, and that for the action of 'immune' body and 'complement' in the case of haemolytic and other cytolytic sera and the cells attacked; and also for the action of ordinary hydrolitic enzymes upon their substrates.

In all the above cases three things are required, viz.:—(1) a body of a ferment nature, (2) a substrate on which it can act, and (3) a body which enables the ferment to act upon the substrate so as to cause hydrolysis, oxidation, or some other type of chemical reaction. Also, the ferment and the substrate are usually much more specific to each other than is the third body, which is simpler in nature, such as an alkali or acid or a peroxide, and we suggest that *complement* is an activating substance of this kind.

Returning to the oxidizing ferments, we may now quote the classification given by Bach and Chodat. In their general review of the subject of oxidizing ferments, published in 1903, these authors give the following list:—

'I. *Oxygenases.* Protein-like bodies which take up molecular oxygen with peroxide formation.

'II. *Peroxidases*, which enormously raise the oxidation power of the peroxides which by themselves oxidize very sluggishly, at the dilutions in question.

'III. *Katalases*, which destroy peroxides with evolution of oxygen.'

In our opinion, all these three names are ill-chosen, because they break the general law that the name should, as, for example, in lactase, tyrosinase and lipase, indicate by the root of the word the substrate acted upon, and by the termination 'ase' the fact that the body designated is an enzyme. Now, in the first two classes named above, oxygen and peroxide respectively are not the substrates, but the bodies which the particular enzymes bring into action upon the substrates, and much more resemble the alkali in trypsin action or the acid in pepsin action. Further, in the third class, the '*katalases*,' there is no reliable evidence of this destructive action upon hydrogen peroxide being due to an enzyme at all. Although it is clearly a distinct activity from that of the oxidizing ferments, yet it is not an oxidation, and it is not specific, occurring, as it does, with every ferment solution of whatever type, with nearly all animal

or vegetable fluids, and with numberless inorganic catalysts. In any case, it is absurd to give it a name which belongs to or includes the whole vast range of catalytic actions. Every true enzyme is a '*katalase*' in the sense that it acts catalytically, and why a catalytic agent, which happens to act upon hydrogen peroxide, and of which it has never been clearly shown that it is a specific enzyme, should be dignified with the name of '*katalase*' it is difficult to conceive. Whatever the body is (or the large number of bodies), it is quite certain no oxidizing enzyme is in question, since nothing is oxidized and the oxygen is simply discharged as molecular oxygen.*

The statement which is made by Bach and Chodat[10] that the so-called oxydases are mixtures of *peroxide bodies* and peroxydases, appears to have led some of their English reviewers to entertain the view that they regard the oxygenases as unstable peroxides rather than as true enzymes producing peroxides. But that this is an error is shown by the above classification, as also by the repeated statement in their works that the oxygenases are ferments which *produce* the organic peroxides, and that it is only in the presence of the peroxides that the peroxidases have any action whatever.

Thus the rationale of the complete reaction of oxidation by these ferments, according to Bach and Chodat, is as follows:—

First an enzyme, called *oxygenase*, acts upon certain substances present in the plant and forms organic peroxides; and, secondly, another and distinct enzyme, *peroxidase*, which is entirely unable to act in absence of formed peroxide now comes into activity and causes a reaction which transfers the oxygen, previously attached to the peroxide by the oxygenase, to the oxidizable substrate, which may be one of several substances.

We have also looked carefully through the later papers by these authors, and have not been able to find any abandonment of the position that the oxygenases are peroxide-producing enzymes.

Thus, Bach[11] states the oxydases are nothing else than mixtures of peroxydases and oxygenases, that is, of peroxide-activating and of peroxide-building enzymes. In this paper, because he obtained by alcohol precipitation, and re-dissolving in water, a solution which alone only slowly attacked tyrosin, but was strongly activated towards tyrosin by addition of small amounts of hydrogen peroxide, Bach claims to have

10. *Bio-chemisches Centralblatt*, 1903, and elsewhere.
11. *Berichte*, Vol. XXXIX, No. 10, p. 2126, 1906.

shown that the usual tyrosinase which attacks tyrosin at once is also a mixture of an oxygenase and of a peroxidase which is specific for tyrosin. No further proof is here given of the enzymic nature of the supposed oxygenase of the tyrosinase.

Again, in 1908, in a polemical paper against Chodat, who had been unable to repeat his observations as to the activation of tyrosinase by hydrogen peroxide, Bach[12] states that the failure of Chodat to repeat his results was due to the use of too high concentration of hydrogen peroxide, and in this paper he once more enunciates that 'tyrosinase, like the 'ordinary oxydases, is composed of an oxygenase, that is, a body which 'produces (" bildet ") peroxide accompanied by uptake of oxygen, and is 'replaceable by hydrogen peroxide, and of a peroxidase, which activates 'the so-formed peroxide, or the added hydrogen peroxide.'

There is proof given by Bach and Chodat that peroxide is present in fresh plant juice which has been treated with air,[13] but none that this peroxide is produced by an enzyme. The proof as to the presence of peroxide, which is not entirely free from experimental suspicion, was obtained as follows. Air was passed through a sample of fresh juice from *Lathraea squamaria* containing oxydase, and at the same time a 1 per cent. solution of barium hydrate was slowly dropped into the juice. A barium precipitate was obtained which did *not* give the hydrogen peroxide reaction with the titanium reagent, after washing and decomposing with dilute sulphuric acid. The solution did, however, intensely blue potassium iodide and starch, and as the absence of nitrate was shown otherwise, it was assumed that an acylated hydroperoxide was present.

It does not appear to us that much that is new is proven by this experiment, since the oxidase known to be present in the original juice would have similarly acted on iodide and starch solution, and there is no reason why it should not be precipitated unchanged by the baryta.

On other grounds it is highly probable that traces of organic peroxides are present in most fresh plant juices. (See Expt. XX.)

This does not, however, prove that they are formed either in the plant or after separating the juice by an enzyme such as the postulated 'oxygenase' of Bach and Chodat, and all experimental proof of such enzymic origin is hitherto lacking.

12. *Berichte*, Vol. XLI, p. 216, 1908.

13. We shall show in our own experiments later that *excess of air* or oxygen, as by standing in shallow layers in open vessels, again destroys the peroxide first formed, so that once more no result is obtained without added peroxide.

Again, the presence of the elusive oxygenase is not proven by the thermal instability of the spontaneous blueing by guaiacum. For the peroxides are also very unstable bodies thermally, and the failure to obtain a blue with juice and guaiacum alone after heating to 50°—60° C., and then obtaining it on adding hydrogen peroxide, may easily be due to the destruction of the thermally unstable organic peroxides, and not to that of an enzyme which produces such peroxides.

In fact, the failure of the blueing after heating is all in favour of the simple presence of unstable peroxides. Because, if it were an enzyme (oxygenase) only that were destroyed by the heat, then this enzyme previously to the process of heating would have had ample opportunity to preform a good supply of organic peroxide, and although the enzyme were destroyed on heating to 50°—60° C., there would be enough preformed peroxide to still give the blueing afterwards.

The simplest hypothesis, accordingly, is that the heating merely destroys the organic peroxides which are essential to the action of the only ferment required, viz., the peroxidase, and on now replacing this loss of organic peroxide by the simple hydrogen peroxide, the blueing is obtained because the essential chemical linkages are present for the peroxidase to act.

In regard to the experimental fact discovered by Bach and Chodat that the juices of certain plants, such as cucumber and radish, do not give a blue with guaiacum alone, but at once give a fine blue on addition of traces of hydrogen peroxide, while other juices, such as those of potato and carrot, give a blue at once, with guaiacum alone, we can completely confirm the experimental observation, but believe it is susceptible of a much simpler explanation than that the potato and carrot contain two ferments, oxygenase and peroxidase, which act as above described, while in cucumber and radish the oxygenase is absent and peroxidase only present.

The oxidation in presence of added hydrogen peroxide is easily demonstrated by means of any of the colour tests, in the many vegetable juices or animal extracts or secretions which contain an oxidizing ferment. But it is an exceedingly difficult matter to trace to their true causes those cases in which a positive result is obtained without the addition of hydrogen peroxide.

Such a positive result is more often obtained when the guaiacum test is employed than with the other reagents we have used for testing. This very frequent positive result is not always due to development of peroxide

in the reagent on standing, as is usually said to be the case, but is present or absent in a most capricious way. In our opinion, the variation in result is due to the presence or absence of preformed peroxide of organic nature in the particular pieces of resin from which the tincture was made, although no doubt there may be a tendency for the amount of this peroxide to increase as the reagent grows older.

The only similar variation with the other test substances we have observed was in the behaviour of potato juice to *p.* phenylene-di-amine in giving the characteristic green without added peroxide of hydrogen when the potato juice was tested immediately after grating the potatoes. Within half an hour the same juice gave not a trace of green until hydrogen peroxide had first been added. Further, even on adding hydrogen peroxide to its juice, if the mixture was left for some few minutes before the *p.* phenylene-di-amine was added no green was obtained. Thus, the peroxide had been destroyed in the meantime, instead of any having been formed as would have occurred if an oxygenase or peroxide-forming ferment had been present.

In this same potato juice, even when quite fresh and giving the *p.* phenylene-di-amine reaction, the positive result of an amethyst colour with α-naphthol could *not* be obtained until hydrogen peroxide had also been added.

With this one exception of the potato juice immediately on preparation, all the colour reagents except guaiacum invariably gave us a negative result until hydrogen peroxide was added. The guaiacum gave us a positive result in the capricious way described above in many instances, such as potato, carrot, wheat, oats, apple, banana, and several species of nuts.

The causes of the variations with the guaiacum will presently be traced out as we followed them up experimentally, but before taking this up we would like to point out that in order to justify the views of Bach and Chodat, that there are two enzymes present, one of which produces peroxides, while the other activates reaction between such peroxides and the oxidizable chromogenic body, the evidence obtainable from the use of different colour-reagents must be consistent and concordant. That is to say, in the same juice one reagent must not indicate the presence of peroxidase only and absence of oxygenase, and another reagent indicate both oxygenase and peroxidase.

Now this is precisely what occurs experimentally, for with the exception of the freshly made potato juice reacting positively to

p. phenylene-di-amine at the same time that it reacted negatively to **α-naphthol,** the evidence was conclusively negative in regard to 'oxygenase' throughout, save for the capricious results with guaiacum only. If now it be admitted that these variable guaiacum results were due to varying amounts of peroxide in the guaiacum, and hence afforded no evidence of 'oxygenase' in the juice being tested, then all evidence for the existence of oxygenase disappears, and we are left with one enzyme, or type of enzyme only, which produces its effect by activating towards each other peroxide and the substrate to be oxidized.

In support of the view that the different behaviour of guaiacum tincture was due to varying amounts of peroxide in the tincture itself, we submit the results of Expt. XX detailed later (see p. 158), which show that when means are taken to exclude peroxide from both guaiacum and juice being tested, a negative result is always obtained until peroxide has been added from outside.

But if this be accepted we are left only with the positive result with perfectly fresh potato juice and *p.* phenylene-di-amine, and since this rapidly disappeared as the juice stood, and, further, as traces of added hydrogen peroxide similarly disappeared on standing, we are disposed to attribute this effect to traces of peroxide in the fresh juice which disappeared on standing.

If such a ferment as the postulated 'oxygenase' had really existed in the potato juice, then the amount of peroxide would not have decreased on standing, but, on the contrary, there would have been an increase all the time, and the test would have grown stronger instead of disappearing. The disappearance was probably due, in part, to the substances ('katalases') present in all such juices which decompose peroxides after the juice is shed from the cells, and in part due to the using up of peroxide in the oxidations brought about by the tyrosinase and other oxidizing ferments which we have been calling peroxidases.

That the rate of disappearance of peroxide is in some way connected with exposure is shown by the fact that the positive reaction with *p.* phenylene-di-amine is lost in a very short time (inside half an hour) when exposed to the air in a thin layer in a flat-bottomed glass dish, while it is still present after several hours in a sample of the same juice preserved in a tightly corked flask which has been completely filled. A positive reaction to a sample of guaiacum tincture which gives no colour with carrot juice is still given by this potato juice, for long after it has ceased to react with *p.* phenylene-di-amine. This is probably due to two reasons:

first, that the guaiaconic acid is more readily oxidized (or at a lower chemical potential) than the *p.* phenylene-di-amine, and secondly, that there is a conjoined effect of the minimal traces of peroxide in both reagent and juice in one case, and of that in juice only in the other.

It would thus appear that all those cases in which a colour test of oxidation is obtained without adding hydrogen peroxide are due to peroxide already present in traces either in the juice or in the reagent. Also, that the amount of peroxide in the juice decreases instead of increasing on standing, and that the peroxide which is specially present in varying amount in the guaiacum reagent exists in the resin and its products quite at the beginning, and so may be present in absolutely freshly made tinctures, from which it may be removed by treatment with charcoal.

It was further found that a mere trace of a reducing agent, such as a drop of dilute ammonium sulphide, or sulphuretted hydrogen water added to half a test tube full of a fresh juice showing the guaiacum test without addition of hydrogen peroxide, was quite sufficient to permanently destroy all the trace of organic peroxide present, so that now the reaction became negative.

RECORD OF EXPERIMENTS

The chief substances used by us for demonstrating oxidation were (1) Guaiacum in fresh 10 per cent. tincture made from the resin. (2) A 1 per cent. solution of *p.* phenylene-di-amine in distilled water. This gives on oxidation a fine green colour, which usually strikes out quite suddenly after a pause of several seconds; it occurred positively only after addition of hydrogen peroxide, saving the exception above mentioned of potato juice, where it is obtainable without added peroxide for a period of about half an hour in the fresh juice; after that it is only obtainable when hydrogen peroxide is also added. (3) A 1 per cent. solution of *a* naphthol in 50 per cent. alcohol. This reagent never gave a positive result with any of the juices, however fresh, until hydrogen peroxide was also added; it strikes a fine amethyst colour in presence of the peroxidase and peroxide; the reagent turns slowly amethyst coloured on standing, and should be made fresh. (4) Indo-phenol or Spitzer's reagent, which we prepared fresh in all cases by mixing equal parts of the two previous solutions of *p.* phenylene-di-amine and *a* naphthol, and of a 2 per cent. solution of sodium carbonate. This on oxidation yields a fine

purple; the test never gave a positive result unless hydrogen peroxide was added, the development of colour being no greater than occurs spontaneously in the diluted reagent or in *boiled* juice of equal concentration, to which the reagent has been added in equal quantity. (5) Hydrochinon in 1 per cent. solution in water. (6) Synthesized 'guajacol' (Merck). This behaves quite differently from the natural guaiacum resin, giving a strong brown colour, and on standing a brown precipitate, obtainable only in presence of peroxide. (7) Guaiaconic acid (Merck) used in 2 per cent. solution in absolute alcohol.

In making comparative tests care was taken to use as nearly as possible corresponding amounts of juice equally diluted, of reagent, and of hydrogen peroxide where that reagent was added. In the earlier experiments a solution of hydrogen peroxide, the usual laboratory strength (10 vols. of H_2O_2 per cent.) ten times diluted was employed, and in the later experiments the pure perhydrol of Merck was used, first diluted ten-fold, as a stock solution made up in small volumes at a time, and this was then again ten times diluted immediately before use.

As a general rule, about 5 c.c. of juice was taken or a given dilution of the juice with distilled water, and to this about 1 c.c. of the diluted peroxide was added and 1 c.c. of the reagent being used.

Where necessary three tubes were used, of which one contained the juice after boiling, another the juice without boiling, with reagent only added, and a third, unboiled also, to which both reagent and hydrogen peroxide had been added.

In other cases where the juice was of known character and the presence of peroxide was not being proven, but rather the effect of reagents upon its activity, this procedure was not necessary, and the experimentation was modified accordingly.

Experiment I.—Testing of *wheat* for oxidizing ferments. One part of wheat by weight, extracted with three parts by volume of distilled water at 30° C., filtered to an almost clear filtrate—water in contact with the powdered grain for about one hour.

Reagent	1. Boiled extract alone	2. Unboiled extract plus hydrogen peroxide	3. Unboiled extract alone
1. Guaiacum	Blue*	Blue	Blue
2. *p*-phenylene-diamine	Nil	Green	Nil
3. *a* naphthol	Nil	Amethyst	Nil
4. Hydrochinon	Nil	Brown	Nil

* This result was afterwards shown to be due to insufficient boiling of the extract.

Experiment II.—Similar experiment with powdered and extracted *oats*, giving exactly similar results.

Experiments III and IV.—*Swiss Condensed Milk* and a proprietory food called *Glaxo* gave negative results on testing with all the usual reagents. This is to be expected, since all such foods and also canned fruits and patent foods for children and invalids are sterilized by boiling. It is a point worth bearing in mind that this absence of oxidizing ferments distinguishes all preserved foods from fresh foods.

Experiment V.—*Fresh milk*, tested as above to guaiacum, *p.* phenylene-di-amine, *a* naphthol, and hydrochinon, gave a reaction only in presence of added hydrogen peroxide, except in the case of guaiacum, where a slow blueing occurred without the peroxide.

Experiment VI.—*Fresh carrot juice* was positive to guaiacum with and without addition of peroxide, but positive to other reagents only after peroxides. In latter experiments with a guaiacum free from peroxide carrot juice was often found negative, especially after standing in air for some time.

Experiment VII.—*Fresh potato juice* was found the most strongly positive of all the juices tested towards guaiacum; no preparation of guaiacum was used throughout which did not give a blue with it, but when both potato juice and guaiacum were deoxidized as much as possible, the blueing effect practically disappeared. Potato juice when just drawn off is slightly positive to *p.* phenylene-di-amine but negative to the other tests, and turns negative to the *p.* phenylene-di-amine also after about half an hour.

Experiment VIII.—Potato juice was dried in an air oven at about 50 to 55° C. and then extracted with water at 48° C. for forty-eight hours. This very materially reduced the oxidizing power, a faint blueing was still obtainable both with and without added peroxide, but stronger with; hydrochinon both negative; *a* naphthol negative without, slightly positive with; *p.* phenylene-di-amine, both negative.

Experiment IX.—*Wheaten flour* gave strong plus to guaiacum with and without peroxide; faint reactions with other reagents and only in presence of peroxide in each case.

Experiment X.—Serum of pig's blood, three days old, gave negative to guaiacum both in presence and absence of hydrogen peroxide; to *p.* phenylene-di-amine slight reaction without and strong reaction with peroxide; to *a* naphthol, peroxide tube strongly positive, doubtful without peroxide.

Experiment XI.—Effect of minute amounts of acid and alkali and of acid and alkaline phosphates on the oxidizing reactions. This was tested as follows, using guaiacum as reagent:—

The acid stops in minute amounts; much more alkali is required to stop the oxidation, but the colour changes from blue to a yellowish green. The tests were carried out with potato and carrot juices:—

Fresh potato juice diluted ten-fold with distilled water, and 5 c.c. of juice taken to quantity named below of reagent and then 0·5 c.c. of tincture of guaiacum added.

1. Normal control. Blue at once.
2. Added 0·1 c.c. of $\frac{M}{10}$ HCl ($=\frac{M}{500}$ approximately). Faint blue, very slowly deepening.
3. Added 0·1 c.c. of $\frac{M}{10}$ NaOH. As blue as control.
4. Added 0·5 c.c. of 5 per cent. NaH_2PO_4 ($=\frac{M}{25}$ approximately) As blue as control.[1]
5. Added 0·5 c.c. of 5 per cent. Na_2HPO_4 ($=\frac{M}{25}$ approximately) Blue much weakened.

1. This reversed behaviour of the phosphatic solutions as compared to the acid and alkali is very peculiar, and difficult to understand, but it was several times observed.

Carrot juice treated exactly the same way gave:—

1. Control. Strong blueing.
2. Added 0·1 c.c. of $\frac{M}{10}$ HCl. No blueing.
3. Added 0·1 c.c. of $\frac{M}{10}$ NaOH. Strong blueing.
4. Added 0·5 c.c. of 5 per cent. NaH_2PO_4. Strong blueing.
5. Added 0·5 c.c. of 5 per cent. Na_2HPO_4. Weak blueing.

Experiment XII.—Fresh juice from grated *radishes* was taken and tested to (1) guaiacum, (2) *p.* phenylene-di-amine, (3) *a* naphthol, (4) pyrogallol, (5) indo-phenol (Spitzer's reagent).

The same result was obtained throughout, viz., negative in absence of hydrogen peroxide; positive in presence of the peroxide. There is absolutely no blueing with guaiacum alone, even with guaiacum which alone gives a good blue with potato juice. On standing over night in a stoppered vessel the same radish juice now gives a fair blue with the same guaiacum. The remaining portion of the radish juice was centrifuged, first alone and then after fractional precipation, with alcohol added up to 25 per cent. in the mixture. It was found that both sediments were very strongly active, much more so than the supernatant liquor.

Experiment XIII.—This experiment was made with *carrot* juice and with *apple* juice. In each case the fresh juice got, as usual, by grating

and filtering was tested alongside an extract made by drying the grated mass at 45° C. for some days. The carrot gratings had been in the oven for three days and the apple gratings for eight days.

The contrast in the filtrates from the fresh and the dried preparations is most striking. The dried is throughout completely negative, and the fresh positive. With guaiacum alone there is little blueing, even in the case of the fresh juices, but immediate effect with addition of peroxide; in neither case is there an effect with the filtrates from the dried materials. With *p.* phenylene-di-amine, *a* naphthol, and indo-phenol there is a very strong reaction, but only in presence of hydrogen peroxide with the fresh preparations, and nothing with preparations from the dried material.

Experiment XIV.—Effects of partial or fractional precipitation of potato juice with alcohol. The properties of the different alcohol precipitates, and results of combustion of the dried alcoholic precipitates.

A quantity of 320 c.c. of fresh potato juice was taken and thoroughly centrifuged. The deposit consisted of starch underneath with a thinner greenish brown layer on top like very fine mud, or ooze. The upper layer could be easily washed off from the strongly impacted starch granules underneath.

It was so removed, shaken up with distilled water, re-centrifuged, and separated again from the small amount of starch mechanically removed with it at the first separation. It was once again shaken up with distilled water, and the brownish colloidal solution or suspension which frothed strongly was found to be strongly active, giving a good blue with guaiacum alone. Examined under the microscope it shows a field crowded with exceedingly minute particles much less than 1μ in active Brownian movement.

This deposit is like an excessively fine mud, which readily passes into suspension: it closely resembles the different fractional precipitates with alcohol, about to be described.

The supernatant fluid after centrifuging was still opalescent and gave a strong blue with guaiacum, to this one-quarter of its volume of absolute alcohol was added, making a 20 per cent. alcoholic solution, in which a copious greyish brown precipitate appeared.

Throughout it was found difficult and tedious to separate this exceedingly fine precipitate by filter and pump, and that they settle excellently and quickly into a compact mass with the centrifuge so that they can readily be separated by decantation.

The precipitate so separated from 20 per cent. alcohol on shaking up

with a considerable quantity of water gives a fine and durable suspension, which gives a fine blue with guaiacum alone and strongly positive tests with the other reagents after addition of hydrogen peroxide. The supernatant fluid is still opalescent and still gives a good blue with guaiacum. A quantity amounting to 350 c.c. of this supernatant fluid was taken, and to it 88 c.c. of absolute alcohol was added, bringing up the alcohol concentration to 36 per cent., giving rise to a considerable amount of a second alcoholic precipitate, which, on separation and shaking up with water, was likewise found to be strongly positive. Separated supernatant fluid again taken and treated with one-fourth its volume of absolute alcohol, so bringing strength up to approximately 49 per cent. by volume; the third alcoholic precipitate was obtained, also giving fine blue with guaiacum alone and positive result with other reagents *plus* hydrogen peroxide. Again added one-fourth volume of absolute alcohol to supernatant fluid, bringing percentage of alcohol up to 60 per cent., and giving fourth alcohol precipitate, which also gives strong positive tests.

Finally, this supernatant fluid, which was now quite clear of suspended particles, had an equal volume of absolute alcohol added to it, so bringing the alcoholic content up to 80 per cent. by volume. A heavy flocculent precipitate was so obtained and separated by centrifuging and decanting. The supernatant liquid now gave no positive tests, but the fifth and last precipitate did give such positive tests after suspending by shaking in water.

All the different precipitates caused by successive additions of alcohol are alike in appearance, all contain oxidizing enzyme, and all give similar composition on combustion; they are, moreover, exactly like the suspension naturally present in the potato juice and partially thrown out by the centrifuge. All the precipitates have the appearance of a fine alluvial mud, and pass up into a fine suspension with fine particles in Brownian motion when worked up into water. This suspension remains permanent over night, and settles very slowly. Notwithstanding this, quite clear solutions yet exceedingly active as oxidizers are obtainable when fresh potato juice is allowed to stand untouched for twenty-four hours, so that there must also be a portion of oxidizing ferment in true solution. It is probable that a part of the fine deposit can pass into solution and show oxidizing properties, for even after thorough washing the suspensions show strong oxidizing properties.

An explanation of the peroxidase results obtainable both with the water clear juice, and with the thoroughly washed colloidal suspension

of the same juice may possibly be that the oxidizing ferment exists in two phases, a portion being in true solution and a portion in excess as colloidal particles in fine suspension.

Iodine gives no distinctive colour, although the suspensions show marked reduction with Fehling's solution.

When the neutral suspensions are boiled there is very marked frothing, the test-tube filling with froth. Part of the emulsion breaks down on boiling, but a good deal still remains as a milky suspension. After just boiling, there is a very slow blueing with guaiacum alone, much increased on adding hydrogen peroxide, and on further boiling for about one minute, all oxidizing power is lost in either presence or absence of peroxide.

Addition of a trace of hydrochloric acid causes no immediate effect, but on standing for a few minutes the emulsion settles completely and the supernatant fluid has lost all trace of oxidizing properties with or without peroxide.

A trace of alkali does not cause loss of oxidizing properties, but a greenish yellow shade appears in the blue colour produced, more alkali inhibits the activity and destroys the ferment.

In a second experiment with potato juice a similar fractional precipitation with alcohol was carried out after all starch had been thoroughly thrown out by prolonged centrifugalization, and then, instead of taking up in water, the several precipitates were dried in a desiccator over sulphuric acid to a constant weight, and the dried scale preparations so obtained were first examined qualitatively by the protein tests, tested for phosphorous and manganese, and a portion was combusted of each in order to ascertain if the composition varied as the percentage of alcohol was increased. Similar testing was also carried out with similar precipitates from turnip and carrot juices.

The following table shows the ultimate analyses of the precipitates caused by the alcohol in the potato juice after drying to constant weight; the first precipitate, with 20 per cent. of alcohol, was too small for analysis:—

COMPOSITION OF DIFFERENT OXIDIZING PRECIPITATES THROWN OUT BY THE GIVEN PERCENTAGES OF ALCOHOL FROM FRESH POTATO JUICE

No. of pp. and percentage of alcohol by volume in fluid from which thrown out	Ash Percentage	Percentages reckoned in ash-free substance of— C	H	N	O, etc.
No. 2. pp. from 36 per cent. of alcohol	7·07	48·9	7·3	10·2	33·6
„ 3. pp. „ 49 „ „	4·71	46·9	7·0	11·1	35·0
„ 4. pp. „ 60 „ „	10·96	46·3	7·2	12·2	34·3
„ 5. pp. „ 80 „ „	29·89	45·1	6·9	9·9	38·1
Average for organic material	—	46·8	7·1	10·8	35·3

It is to be observed that while the ash varies considerably, the composition of the ash free organic matter remains fairly constant. The composition approaches more nearly that of the mucins or gluco-proteins than that of any other class of bio-chemical bodies. In this connection it is noteworthy that the suspensions in water of all the precipitates very strongly reduce Fehling, and they are also thrown down as above stated by very dilute acids, and even the less strongly ionized organic acids, which destroy their oxidizing activities. On the other hand, as we shall point out, the precipitates either fail altogether with certain of the protein tests, or in other cases indicate traces only of proteins.

The ash always contained manganese, as shown by Bertrand, and iron in small amount, and phosphates in fair quantity were invariably present after incineration and applying the molybdate test.

As a general rule, a biuret test could only be obtained after boiling, and then only a trifling show of colour, nothing whatever of the colour given by free protein being seen in the cold. The xanthoproteic test was negative in the cold, and after boiling only the palest yellow was obtainable, but a certain amount of deepening towards an orange colour was obtained on adding ammonia in excess. The Millon's reagent gave quite negative results throughout.

Our general impression from the qualitative protein tests is that protein is absent in these highly active precipitates, although there is a high nitrogen percentage, as shown by the above figures, which indicate a carbohydrate moiety joined on to a radicle rich in nitrogen but not containing the groups which are present in ordinary protein and give the colour tests.

In order to find out whether prolonged drying to a constant weight over sulphuric acid in the desiccator had any effect upon the oxidizing effects, the remaining portions of the four dried precipitates which yielded the analytical results given above, and which had been kept dry from November 11th till November 26th (fifteen days), were taken and ground up in distilled water. All yielded again very fine emulsions like those originally obtained, showing exceedingly minute particles, under the one-sixth objective, in active Brownian movement. The only change from the original condition is that now scarcely any blueing is obtained with the guaiacum test until hydrogen peroxide also is added, when a fine blue is obtained; this shows that all the preformed peroxide of the fresh juice has disappeared, but that the peroxidase enzyme is still present and active and ready to induce interaction between substrate and peroxide. The

other colour tests are, as usual, completely negative in absence of hydrogen peroxide, and strongly positive in its presence.

Carrot juice behaves much like potato juice in regard to fractional precipitation by alcohol, all the precipitates being less in amount from the same amount of juice, and all being active; the 80 per cent. alcohol finally left after precipitation is inactive. With turnip juice nearly all the oxidizing ferment comes down in the third fraction (57 per cent. by volume), and the 80 per cent. supernatant fluid is clear and free from oxidizing action. The precipitates by showing a reducing action on Fehling, and by failure to give protein colour tests, behave exactly identically with the potato juice precipitates.

Experiment XV.—Effects of exposure to oxygen, and of shutting off from air, or of exposing to air in shallow layers respectively.

Here we came across one of the most puzzling and difficult to understand of all our results. So far as we are able to follow them, there appear to be two opposed effects, one of a more rapid formation of peroxide *in very small amount* from some precursor in the very fresh juice, and the other of a slow breaking up of the preformed peroxide which is slowed or avoided in tightly sealed up full vessels.

For example, a freshly cut surface of cucumber, radish, carrot, or certain other vegetable tissues shows no blueing with a guaiacum preparation which is itself free from peroxide either naturally or by special treatment. (See Expt. XX.) But merely grating the cucumber to get the juice and filtering from débris of tissue through a clean cloth is enough to give origin to a certain amount of blueing power in absence of added hydrogen peroxide. Further, vigorous shaking up of this juice with air, or bubbling oxygen through it for some minutes, materially increases the amount of natural peroxide, and hence the blueing effect with guaiacum alone. Now, if the juice be separated into two parts, one of which is used to fill a small bottle, which is then tightly corked, and the other is spread out in a thin layer on the bottom of a Petri dish, then, entirely contrary to what might have been anticipated, the tightly corked up sample juice will be found after a few hours, or even after a couple of days, to give a good blue with guaiacum alone, while the juice exposed to the air will probably have lost all blueing power except on addition of hydrogen peroxide. This change occurs probably in a few hours, certainly within twenty-four hours. Hence, in the early stages shaking with air develops peroxide, but the peroxide formed is unstable in presence of atmospheric oxygen, which ultimately robs the juice of all its small store of peroxide.

Not only can the exposure destroy the natural peroxide, it (or some peroxide-destroying enzyme such as catalase present in common solution) can also destroy added hydrogen peroxide, for if to a juice which is not giving an oxidizing reaction with guaiacum alone one adds a minute amount of hydrogen peroxide, sufficient to give a good immediate blue on further addition of the guaiacum reagent, and if now the juice so treated be divided into two equal parts, to one of which guaiacum is added, at once giving a good blue, and the other allowed to stand for five minutes, then it is found that no colour is given by the latter owing to destruction of the added peroxide in the interval.

That this is the true explanation is shown by the immediate blueing obtained on adding more peroxide after the guaiacum has failed to give the colour in the second portion.

By taking a large quantity of juice, adding small quantities of hydrogen peroxide at a time, and testing small portions at intervals after each addition of peroxide, this peroxide destruction may be followed out as often as desired.

This experiment further demonstrates that there is no firm linkage between ferment and peroxide such as can prevent the latter from destruction, for then enough peroxide would be permanently preserved linked on to the ferment to give a blue colour at once when the substrate (guaiacum) was added even after a time interval.

Experiment XVI. Effect of reducing agents upon ferment and oxidizing reactions.

The oxidizing ferment is extremely susceptible to the merest traces of reducing agents, such as sulphuretted hydrogen or ammonium sulphide, for the addition of a drop of diluted ammonium sulphide or sulphuretted hydrogen water to a half test tube full of potato juice will not only stop blueing with guaiacum alone, but will prevent it even in presence of a large excess of hydrogen peroxide. The ferment appears to be completely and irrecoverably destroyed, either by molecular change or by firm anchorage of the sulphide. At any rate, we have never been able to restore activity once the reducer has been added.

Experiment XVII. Thermo-labile nature of the peroxides.

Except that we prefer to call it peroxide instead of oxygenase, we can experimentally confirm the statements of Bach and Chodat, that the substance giving oxidizing reactions without added hydrogen peroxide is less stable than the ferment or peroxidase which gives rise to the oxidation in presence of peroxide. We have pointed out that our dried precipitates

from alcohol had lost practically all their peroxide, although they had plenty when freshly thrown down and at once taken up in water. Heating to about 55° C. destroys the unstable peroxide but leaves the peroxidase; this occurs more quickly in some juices than in others. Thus, potato juice takes some hours, and even then although the amount is very greatly diminished a slight trace remains which is most difficult to get rid of. For example, a portion of freshly prepared potato juice was divided into eight similar portions, which were placed in an air oven kept at 55° C. on February 7th, 1909, at 12-20 p.m., and the tubes were tested one at a time at the following intervals afterwards:—Tube 1, tested at 12-45, still gives intense blue immediately; tube 2, at 1-20 p.m., blue not so intense and comes more slowly; tube 3, at 2-20, more slowly still; tube 4, at 3-50 p.m., still a good deal of blue, partial precipitation by the heat; tube 5, at 4-30 p.m., still blue; tube 6, at 11-40 a.m. (February 8th), very faint blue only coming after prolonged shaking; tube 7, at 3-40 p.m., no longer gives blue until hydrogen peroxide has been added. Thus a period of about twenty-seven hours was required to destroy all the natural peroxide at 55° C.

The same result occurs more slowly, as pointed out, on standing in air at ordinary temperatures, and here again potato requires longer than any other juice we have experimented with, taking several days, while carrot juice will be almost free within twenty-four hours. We have not investigated whether this is due to a larger original supply, or to slower reduction or greater resistance of the natural potato peroxide. Great variations are experienced of a perplexing nature both as to the rate at which the maximum amount of peroxide is developed, and the rate at which it disappears, for which we are quite unable to account.

Experiment XVIII. Effects of germination on distribution of oxidizing ferment and peroxide.

A small quantity of oats, from the same sample as the grain used in Experiment II, were sown on moist cotton wool, and left in the incubator to germinate from November 4th till November 16th, being kept moist during the period and at a temperature of 32° C. At the end of the period the sprouts were about 10 centimetres long. They were pulled out from the oats, and the sprouts and residues of seeds were tested for oxidizing ferment with the following results:—

Both gave a positive result with ordinary guaiacum tincture alone; both were quite negative to all the other coloured indicators for oxidation until hydrogen peroxide had also been added, and both contained

'katalase,' as shown by vigorous discharge of oxygen when added to hydrogen peroxide (5 vols. per cent.).

Experiment XIX.—Absence of oxidizing enzymes in the fresh juice of lime, lemon, and orange; but presence of such in seeds of lemon and orange.

One of our incentives in commencing this research on oxidases was the necessary absence of oxidizing enzymes by reason of the sterilization, or other form of preparation, from all preserved foods, vegetables, and fruits, as also from sterilized milk and milk substitutes, such as children's and infants' food. While such enzymes are present, and in considerable quantity, in fresh vegetables and juices, in fresh fruits eaten uncooked, and in fresh unboiled milk.

This is a difference of a tangible nature in the chemistry of food, of these two classes, and it appeared possible that it might give some basis for a better understanding of the etiology of such diseases as rickets or scurvy, the former of which is said to be associated with exclusive use of boiled and proprietary foods, while the latter, which is, however, a distinct condition, appears to arise from prolonged abstention from fresh vegetable food.

It was these considerations which led us to try those antiscorbutic vegetable juices which are accredited with the most powerful properties, such as lime juice, lemon juice, and orange juice.

We were greatly surprised to find that this group amongst the many which we tested was the only one which did not yield good oxidase reactions either in presence or absence of peroxide. Entirely negative results were given both by juice and rind, and the crushed seeds only in the case of lemon and orange (we were unable from lack of material to test the lime seeds) gave a somewhat feeble positive effect.

The absence of oxidase in the juice of the fruit of this group is very interesting, although we are at a loss at present to account for it. The peroxidase is not simply concealed by the somewhat high acidity of the juices, for no more success is obtained in the testing on neutralizing the juice. It may be that the organic acids present inhibit the production of the ferment, as they certainly inhibit its action when added from without, and tend to destroy it. Thus, if a quantity of potato juice be mixed with the acid (or even almost neutralized but still faintly acid) lemon juice, on now adding guaiacum a negative result is obtained; but if the acid be completely neutralized the usual blueing follows, especially on adding hydrogen peroxide.

In testing the lime juice care must be taken to use a sample which

has not any sulphurous acid as preservative, as this gives a transient oxidation and blueing with guaiacum even in minimal amounts.

The crude juice gives not a trace of effect with any of the tests for oxidizing enzymes.

Experiment XX. Direct application of guaiacum test for peroxidase and peroxide, to fresh cut surface of vegetables.

This was carried out by slicing, after thorough washing of the outer surface, and then applying an 'old' solution of guaiacum to one surface and a 'new' solution of guaiacum to the other surface. The test is a convenient one for peroxide and peroxidase distribution in different parts of the tissue, and also demonstrates that even in those plants which yield most easily the reaction with guaiacum alone, such as the potato and carrot, the natural organic peroxide is not present as such in the plant tissue, but is formed in the first few minutes after cutting from some precursor in the plant juices.

It may be pointed out that although the terms 'old' and 'new' are used in describing the results of this experiment, this is done because at the time we thought the difference was due to age of the two tinctures. Later we found (see Expt. XXI) that the amount of peroxide present in any given sample of guaiacum was more an accident of amount of impurities of vegetable origin in the piece of resin from which it was originally made up. Accordingly 'old' means containing more peroxide and sufficient to give direct test in presence of peroxidase, and 'new' means comparatively peroxide-free, and hence enables to give blueing with peroxidase in absence of added hydrogen peroxide.

A potato was taken, washed thoroughly, dried, and sliced through with a clean knife. *At once* tincture of guaiacum was placed on the two cut surfaces; 'old' guaiacum on one, and 'new' guaiacum on the other. The surface exposed to the 'old' guaiacum blues instantly all over; that with the 'new' guaicum only at one or two spots and much more faintly. The blueing with the 'new' tincture occurs more especially at a bruised spot or just close to the epidermis. On cutting away about a centimetre all around the peel to remove any injured portions, only a very slight blueing is obtained, for a few minutes, but comes on standing.

In order to test whether the residual amount of blueing in the case of the potato were due to age, as the potato had been removed from the earth for some days or weeks, and since quite negative results had previously been got with fresh radish and fresh cucumber treated with 'new' guaiacum tincture, the following similar experiment was next tried.

A fresh clean carrot was taken, a sectional cut made across it, and the two clean cut surfaces were treated one with 'old' and the other with 'new' guaiacum.

The 'old' tincture caused instant blueing, the colour being most marked for about a zone of half a centimetre thickness round the outside, and again most marked in the protoxylem, the central part is less intensely stained, but the whole surface is still distinctly blue.

The 'new' tincture, on the contrary, gives for over ten minutes' time after first application no blueing except at two minute spots where there happen to be old bruises just under the skin; [14] at about the expiry of ten minutes a faint blue appears as a ring sending out radiating branches, or veins distinctly marking out the protoxylem. It is noteworthy that this is the part of the section where most oxygen would probably be present in the plant tissues.

On adding to the section of fresh carrot treated with 'new' guaiacum tincture, a minute trace of hydrogen peroxide, by touching with a fine glass rod moistened with the diluted peroxide, there is produced at once an intense blueing, showing the failure to obtain blueing initially is due to absence of peroxide and not to absence of ferment.

Experiment XXI.—Effects of destruction of peroxide in both reagent (guaiacum tincture) and vegetable juice by treatment with animal charcoal before use.

This experiment was conducted in order to make a closer study of certain results of the previous experiments which show rather perplexing variations in behaviour towards the guaiacum test (*a*) with different types of vegetable juice, or the juice of the same origin with the age of the juice, and (*b*) taking the same juice and at the same time, variations according to whether one or another tincture of guaiacum made from the same stock of the resin was employed for the test.

With the single exception of potato juice, *for an hour or so after it is made*, giving a positive result with *p.* phenylene-di-amine without addition also of hydrogen peroxide, all the oxy- and amido-phenol coloured indicators of oxidation had concordantly given that a ferment peroxidase alone was present, which required to have peroxide added in order that it might act.

The guaiacum test, on the other hand, as above indicated, gave most

14. A similar application to a section of an apple showing a bruise did not, however, demonstrate any more marked blueing in the neighbourhood of the injured and browned cells. Still, the browning naturally occurring must be an oxidizing effect upon tyrosin or similar chromogenic substances giving colour on oxidizing.

perplexing variations. Thus, even with fresh guaiacum and potato juice as fresh as it can be separated from the grated tuber, a considerable blueing was obtained; fresh carrot under like conditions sometimes gave a blue fainter than that with the potato, sometimes nothing; and fresh cucumber or radish juice, even with moderately stale guaiacum tincture, gave but a poor effect and nothing with fresh tincture.

The most feasible explanation of these peculiar variations which occurred to us, and that which suggested the following experiments, was that there are two natural sources of peroxide, viz.:—(*a*) the plant juice, and (*b*) the tincture of guaiacum. The summation of these two effects causes the guaiacum test to be positive, especially with a strongly peroxide containing tincture, in cases where the small amount of peroxide in the juice is insufficient yet to start the oxidation of the oxy- and amino-phenols. In further support of this, the potato juice, which of all the juices we examined is most strongly positive to guaiacum alone, reacts positively, when it is quite new and highest in its content of natural peroxide, to *p.* phenylene-di-amine, but just fails to appreciably quicken the oxidation of *a* naphthol, unless hydrogen peroxide be added.

We hence had to examine separately the tincture and the vegetable juices, using mildly destructive agents for peroxides which would not destroy the ferment also. Direct reducing agents, such as sulphuretted hydrogen or ammonium sulphide could not be employed, since, as above stated, they appear permanently to destroy the ferment also, either by altering its constitution or by firmly linking on to it.

An attempt to clear the guaiacum of colour by means of animal charcoal although it failed in its immediate objective, giving a much deeper green coloured solution, happily had the advantage of destroying the peroxide of the tincture, and yielding a clear filtrate which had no power of blueing until hydrogen peroxide was also added.

The usual explanation of the variations in peroxide content of guaiacum tincture is that the peroxide is formed as the tincture stands, and hence, for purposes of the test, that freshly made tinctures must always be employed.

While we are not prepared to deny that peroxide may be so formed, as the tincture stands and darkens in colour in the course of several weeks, we have clear evidence that this is not the main source of the variations, and that a quite freshly made tincture just filtered off may give marked blueing, while another sample of tincture a week old may give quite a negative result, although it was made from some pieces of the same stock of resin.

For example, we had in hand two samples of guaiacum tincture, both made with the same absolute alcohol in 10 per cent. solution from the same stock of guaiacum resin, but one was made about ten weeks previously and the other four days previously. The 'old' tincture gave fine blue with all our vegetables on the cut surfaces, as detailed in Experiment XIX, and with the separated juices, while our 'new' tincture gave scarcely a trace of effect. In order to make sure that it was a question of ageing and accompanying peroxide formation, we determined upon making an absolutely fresh preparation, and for this purpose we took some pieces of the guaiacum resin, and washed the outer surface carefully with alcohol in order to remove a green powder which forms on the other surface of the broken guaiacum, and which we thought might be oxidized. These washed pieces were dissolved as far as they would dissolve to make a 10 per cent. tincture in previously boiled absolute alcohol, and a clear filtrate was obtained and used immediately for testing.

To our great surprise, this gave at once a fine blue immediately with the sections of vegetables and juices without adding any peroxide whatever.

We therefore had now a tincture just made which reacted positively without added peroxide, a four days old tincture made without any special precaution which acted negatively under like conditions, and a ten weeks old tincture which behaved exactly like the just made one.

Further, a tincture made from the green powder on the outside of the pieces of guaiacum gave, like the four days old tincture, a negative result.

It was also noticed that while the ten weeks old tincture and the 'new' tincture possessed the deep yellow brown colour of guaiacum tincture, the four days old tincture was much paler in colour, and the tincture made from the surface powder was green in colour. This led us to boil up some of the 'old' tincture with animal charcoal, in order to try to decolourize it, when we found that instead of decolourizing it went darker, and the filtrate had a green colour.

This filtrate tested now upon carrot juice gave no blueing until hydrogen peroxide was added, showing that its peroxide had been removed by the charcoal; it still, however, gave a blue with potato juice alone. Boiled up once more with charcoal, and again added to potato juice, it still caused blueing, but only slowly and less intensely. This final amount of blueing was probably due to the peroxide in the potato juice itself (*vide infra*).

On examining now the residue left behind on the filter paper in filtering the alcohol extract of the guaiacum resin before the addition of animal charcoal, the chief source of the peroxide of the guaiacum tincture, and the cause of the variations was discovered.

The greater part of this residue consisted of broken seeds and tests of seeds, and other vegetable material. These impurities evidently had been collected with the resin, and since, like all such fresh vegetable material they would contain peroxides, it can easily be understood that they would yield peroxide to the tincture. Also, in making up tinctures from the same stock, the amount of such impurities varying in different pieces, would explain the variations in degree of spontaneous oxidizing power shown by the different tinctures in absence of added peroxide. This all the more so because the pieces of seed are quite large, and whole seeds even were seen, much larger than barley seed. It was on this account, in all probability, that the green outside powder was free from peroxide, and by accident, probably, some pieces more free than usual had been picked up for the preparation of the four days old ('new') tincture.

We would accordingly recommend that in preparing a guaiacum tincture for peroxidase experiments this débris be avoided, and that the filtrate be thoroughly boiled with animal charcoal and filtered. Under such conditions the filtrate can be safely used for several days at least, and any spontaneous blueing observed may be certainly set down to the action of peroxide in the juice or vegetable tissue being tested, and not in the guaiacum itself.

The view that the animal charcoal acts by destroying peroxide in the guaiacum is supported by the fact that animal charcoal added in small amount to diluted (1 in 10) hydrogen peroxide solution causes an immediate effervescence.

Having ascertained that the peroxide could be discharged from guaiacum tincture by animal charcoal, we turned the method upon those vegetable juices giving blueing alone, such as potato and carrot, and found that here the peroxide could also be removed by treating in the cold with animal charcoal and filtering.

Carrot juice, potato juice, and a sample of tincture of guaiacum which gave a good blue with each of them without hydrogen peroxide, were severally treated at laboratory temperature with animal charcoal and left to stand for one and a half to two hours, then they were filtered, the guaiacum having turned green in colour.

The carrot juice so treated when now tested with the ordinary untreated guaiacum gives a dirty greyish green instead of the previous deep blue, whereas with the charcoal treated guaiacum it no longer gives a trace of blue or green. Here peroxide has been completely destroyed both from juice and reagent.

The potato juice which before gave an intense blue when now, after charcoal treatment, tested with the ordinary untreated guaiacum gives a dirty greyish blue, and when both charcoal treated potato juice and charcoal treated guaiacum are used only a grey with the faintest suggestion of blue is obtainable.

This experiment, therefore, supports the view that a positive result with the guaiacum test in the absence of added hydrogen peroxide, indicates traces of peroxide either in the guaiacum or in the plant or other juice being tested. By the charcoal treatment the guaiacum can be freed of peroxide, and then the test may be utilised as a fairly delicate one for peroxides naturally occurring in plants, or formed soon after the juice is set free.

Experiment XXII.—Dialysis in parchment paper.

A quantity of 500 c.c. of fresh carrot juice was placed in a sausage tube of parchment paper, and dialysed against 1,500 c.c. of distilled water in a tall cylinder. Next morning the outer fluid gave with guaiacum (ordinary) alone a slow but distinct blueing. This subject, however, requires further investigation.

Experiment XXIII.—Supplementary experiment on presence of oxydases in bananas, in certain species of nuts, viz., Spanish chestnut, almond, filbert, walnut and Brazil nut and in hyacinths.

None of these gave positive results with the phenol or amido-phenol derivatives, or with guaiacum free from peroxide, except when hydrogen peroxide was added, when they reacted in varying degree.

In the case of the banana, the inner surface of the peel rapidly blues with peroxide containing guaiacum, but the pulp only blues very slowly, and the reagent applied to the cut surface shows marked veining accompanying the course of vessels.

Cross sections of a Spanish chestnut, tested at once with both peroxide containing and peroxide free guaiacum, show blueing with the former at once, but no blueing with the latter, even after ten minutes. The blue first produced by the peroxide containing guaiacum bleaches in a few minutes, but can be brought out again even more strongly by re-applying more of the reagent; this is repeated several times. On touching a spot

on one of the sections treated with peroxide free guaiacum with a glass rod previously dipped in dilute hydrogen peroxide solution, there is an instant blueing at this spot.

Brazil nut gives exactly similar results. Almond, filbert and walnut give no colour with peroxide free guaiacum. Walnut and almond give a slight colour, filbert rather more colour, with peroxide containing guaiacum. Haricot beans, old and very dry, gave no colour either with peroxide containing or peroxide free guaiacum, but an extract gave a feeble positive effect with ordinary guaiacum alone after vigorous shaking, heightened by adding hydrogen peroxide.

The green leaves, bulb, and rootlets of a hyacinth, taken fresh from the garden were examined with peroxide free guaiacum, and only the rootlets gave a rather slow blueing with this alone, but all three parts gave a fine blue on also adding hydrogen peroxide.

Experiment XXIV.—Effect of synthetic guaiacol (Merck).

This reagent behaves quite differently from the natural resin, giving a deep brown colour in oxidation which is very distinctive. Tested with fresh potato and carrot juice it gives no effect until hydrogen peroxide is also added, when the mixture at once turns brown, which deepens in colour to reddish brown, and a reddish brown precipitate is thrown down.

Experiment XXV.—Effect of guaiaconic acid (Merck).

This substance is contained in guaiacum resin and turns blue on oxidation. A supply obtained from Merck was found to dissolve completely to a brown solution in absolute alcohol. A 2 per cent. solution was used for testing the fresh juice of potato, carrot, apple, and cucumber. The colour obtained was identical with that given by the tincture of the resin, and, as the following results show, it contained a trace of peroxide, about equal to that in the best of the tinctures made from the resin, and distinctly greater than the tincture when freed from peroxide by thorough treatment with animal charcoal.

Potato juice gave with guaiaconic acid alone a greyish blue, deepening on standing; on the addition of a few drops of diluted hydrogen peroxide an immediate blue was obtained, rapidly deepening on standing.

Carrot juice gave practically a negative result with the guaiaconic acid alone, there being only a slight dulling of the natural carrot colour and no trace of blue in fifteen minutes; addition of a few drops of dilute hydrogen peroxide to half of the test gave an instantaneous deep blue.

Apple juice gave with hydrogen peroxide a dirty greyish green, slowly blueing on standing: on adding peroxide also the usual deep blue was obtained.

Cucumber juice gave with guaiaconic acid alone a faint greenish yellow, increasing and showing just a shade of blue on standing: addition of hydrogen peroxide also caused a deep blueing at once.

THE PARALLELISM IN MODE OF ACTION BETWEEN HYDROLYTIC ENZYMES, OXIDIZING ENZYMES, AND THE ACTIVE BODIES DEVELOPED IN IMMUNE SERA

The experiments recorded in the previous section appear to us to demonstrate clearly that the whole difference between the various juices and other fluids showing an oxidizing action consists in the presence of a variable small amount of peroxide which is chemically unstable and destructable by the agencies recorded.

All the juices showing oxidizing properties possess one type of ferment which, since it acts only in presence of either naturally present or artifically added peroxide, may provisionally be styled a peroxidase, and there is no proof of the existence of any other type of enzyme engaged in oxidation processes.

This not only materially simplifies our conceptions regarding the oxidizing ferments, but, in our opinion, brings the class into line both with the great division of hydrolytic ferments engaged in the processes of digestion and metabolism and with the active bodies in the natural and immune sera which combat and immunize against disease. So that the oxidizing enzymes form a connecting link between the two classes.

In all three classes of enzymic action it is to be observed that three interacting bodies are required. These three are (1) the substrate on which the ferment is to act, (2) the body which is to be combined directly or indirectly with the substrate and alter its chemical and physiological properties, and (3) the enzyme or ferment which is to activate the reaction.

In the case of the ordinary hydrolytic enzymes these are as follows: *Substrate*, the foodstuffs, protein, carbohydrate, or fat; the *Combining Body*,[15] the elements of water finally, intermediately the acid or alkali, in presence of which alone the ferment is active; the *Catalyst*, one of the digestive or other hydrolytic ferments, such as pepsin, trypsin, diastase, zymase, lipase, &c.

15. No generic name, so far as we are aware, has yet been given to this substance usually of a simpler nature than the substrate, which is added to or taken away from the substrate in a catalytic action. We suggest that it might conveniently be called the Combinate

In the case of the oxidizing ferments: *Substrate*, the oxidizable substance, such as tyrosin, naturally occurring phenols in the plant, or the chromogenic indicators used in the preceding section; the *Combining Body*, oxygen yielded by peroxide bodies present in some form, either as simple hydrogen peroxide or as organic peroxides; the *Catalyst*, the enzymes, such as tyrosinase, and the peroxidase experimented with in the preceding section.

In the case of the immune sera, cytolysins, etc.: *Substrate*, the cell or bacterium to be dissolved or the toxic or foreign substance in the serum to be attacked and rendered inert; the *Combining Body*, the complement, or thermo-labile substance, in the absence of which the reaction cannot proceed; the *Catalyst*, the specific immune body or anti-body which attacks and disintegrates the foreign cell or 'neutralizes' the toxic substance.

Between two of these three reacting substances, viz., the substrate and catalyst, there exists usually a considerable amount of specific relationship. This specific relationship is in most cases not narrowed to a single chemical substance, but is closely confined to the members of a class of bodies possessing in their molecular constitution a certain definite grouping. Even very nearly allied groupings are quite inert to the particular catalyst, as, for example, the fermentation by organisms of certain sugars or other substances, which are stereo-isomers of others which are not attacked in the least. But given the *identical* molecular conformation at a certain portion of the molecule, there may be, and will be, attack and chemical action, although the molecular structure at other parts may be such as to render the two bodies in other respects very different physically and chemically.

For example, pepsin and trypsin attack all classes of proteins, down to certain well-marked stages of hydrolytic cleavage so long as certain connections in the molecular aggregate exist, although in physical properties, in reactions to precipitates and indicators, and even in ultimate chemical composition, these proteins are very distinct from one another.

Similarly, the peroxidase ferment in presence of peroxides attacks a large number of oxidizable substances, such as those experimented with in the preceding section, but leaves other classes of oxidizable substances, both those which are more readily and those which are more difficultly oxidizable, unattacked.

Turning to the third member of the group of three, represented by

the alkali or acid in the case of the hydrolytic ferment, by the peroxide in the oxidizing ferments, and by the complement in the immune sera, we find that this is much less specific in character. Thus, an immune body or cytolysin of very specific character may be activated by practically any serum, including the natural serum of the same species as the animal which had been immunized. Similarly, any body containing a peroxide linkage, organic or inorganic, will activate a peroxidase, and any type of acid or alkali which increases hydrogen or hydroxyl ion concentration, respectively, will activate a hydrolytic enzyme.

The ferment character of the reaction in the case of immune sera is also shown by the disproportionately large amount of complement from any normal serum which can be bound to lipöid, and so rendered inert for haemolysis in the second stage of the Wasserman reaction, by very small amounts of syphilitic 'immune' body. In this reaction it is clear that an active principle in the syphilitic serum acts as a catalyst or ferment, the lipöid from liver or elsewhere as a substrate, and the complement from any serum as a combining body with the substrate under the influence of the catalyst.

Finally, attention may be drawn to the similarity between the oxidizing ferments and immune sera in regard to thermo-stability. When an immune serum is heated for some time at 55° C. the complement is destroyed, but the 'immune' body remains untouched; the heated serum, however, is inactive until complement is added by mixing with some unheated serum which may be drawn from any animal.

Quite similarly, when a vegetable juice is heated to 55° C. the peroxide is destroyed, but the ferment or peroxidase is untouched, and although the heated juice is inactive as an oxidizing agent it is at once re-activated on adding hydrogen peroxide or other form of peroxide.

Again, if serum be preserved unheated it slowly loses its complement, and is inactive until fresh complement from an inert serum is added. Similarly, if a vegetable juice be kept it loses gradually its spontaneous power of oxidizing, but regains it as soon as a peroxide is added.

ON THE OCCURRENCE OF A MON-AMINO-DIPHOSPHATIDE LECITHIN-LIKE BODY IN EGG YOLK

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In making some investigations on the ethereal extract of egg yolk, I succeeded in separating a body of the general nature of a lecithin, but containing in its molecule two parts of phosphorus to one part of nitrogen.

This compound is of the same type as that separated from heart muscle by Erlandsen,[1] and called by him 'Cuorin,' though, as shown below, it differs in certain respects from this substance. A preliminary note giving the N and P content which characterise it as a mon-amino-diphosphatide was published some time ago,[2] but as this portion was prepared from a comparatively small number of eggs, no account of its properties or probable elementary composition was given.

During this winter, however, I have isolated the substance from a large quantity of egg yolk, and having used certain modifications in the preparation of different portions, will now proceed to give a short account of its isolation, composition and chief properties.

Preparation

The egg yolk of fresh eggs was separated from the white portion, and after being spread out in thin layers on a glass plate was dried by means of a current of air generated by a fan-like arrangement attached to a motor. When thoroughly dry the mass was broken up into small pieces, and finally passed through a coffee mill. In this way a very fine powder was obtained, which was carefully extracted five times with ether. The ethereal extracts were mixed together, evaporated to a fairly small volume, and treated with acetone. The precipitate was dried in vacuo over H_2SO_4 and divided into two parts. Portion A was treated by a combination of the methods used by Stern and Thierfelder[3] for purifying lecithin, and by

1. *Zeitschrift f. physiol. Chemie*., Bd. LI, S. 92.
2. *Ibid*., Bd. LVII, S. 304.
3. *Ibid*., Bd. LIII, S. 370.

Erlandsen[4] for the isolation of cuorin; as will be seen, this combination is rather tedious in carrying out.

Portion B was treated by a much simpler method, which was found exceedingly easy to carry out, and capable of giving quite a pure substance.

Treatment of Portion A

This portion was again dissolved in ether and gave a markedly turbid solution. By means of the centrifuge a perfectly clear fluid was obtained, the residue in the centrifuge tubes being of a whitish colour, and with difficulty soluble in ether. The clear solution was again precipitated with acetone and dried. This process of purification was repeated five times, and by this means impurities such as cholesterin and fat were in great part got rid of. The final material gave a perfectly clear solution in ether, but only after standing for some time, being at first slightly turbid. The solution was now treated with about four times its volume of absolute alcohol, and left to stand under CO_2 in a closed vessel for twenty-four hours; an alcohol insoluble residue remained, which was separated by filtration. This substance was washed with cold alcohol, and the united filtrates evaporated in vacuo to a small bulk, precipitated with acetone, and dried as usual. This precipitate was now treated with absolute alcohol, when a small portion remained behind, which was added to the above alcohol insoluble part. Thus, the ethereal solution was divided into two parts—the part soluble in cold alcohol consisting of 'lecithin.'

The alcohol insoluble part was now placed in an incubator and heated for a fairly long time with alcohol at 65° to 70° C. By this means part of the substance went into solution, but separated again on the alcohol cooling. This process was repeated three times, and finally boiling alcohol was used in an attempt to obtain an alcoholic solution that would remain quite clear after cooling. In every case, however, the alcoholic filtrate on cooling became slightly opalescent, though only faintly so. By this procedure the part insoluble in cold alcohol was divided into two parts, one of which (portion *a*) was soluble, the other (portion *b*) insoluble in hot alcohol.

Portion (*a*) was thoroughly washed with cold alcohol and treated as described later.

Portion (*b*) was dissolved in ether and left to stand over night under CO_2; next day a slight precipitate had settled out, the solution itself being

4. *Loc. cit.*

almost clear. On centrifuging, the perfectly clear solution obtained was precipitated by acetone; on again dissolving in ether it gave practically a clear solution. This solution being again precipitated, the substance was dissolved in hot ethyl acetate, out of which it separated on cooling. It was then filtered, dried and analysed.

Treatment of Portion B

This portion was thoroughly extracted with cold absolute alcohol, whereby the greater part went into solution. This solution was evaporated to a small bulk and the syrupy residue dissolved in ether; the ethereal solution was then precipitated by acetone. The precipitate was dried, redissolved in ether and reprecipitated by acetone. By this means ordinary 'lecithin' was obtained in a pure condition, the ordinary contaminating substances present in the raw ethereal extract mass being relatively insoluble in cold alcohol.

Residue insoluble in cold alcohol was now treated with hot alcohol exactly as described under Portion A. By this means impurities such as cholesterin and fat went into solution. This treatment with hot alcohol was repeated four times, boiling alcohol being ultimately used. The residue was then further purified by dissolving in ether, precipitating with acetone, and finally dissolving in hot ethyl acetate, as described above; it was then dried and analysed.

The hot alcoholic solution on cooling deposited a voluminous precipitate of floccular masses. This was obviously composed in great part of fat and cholesterin, and was not examined.

This method of separating the raw material is exceedingly simple and quite efficient. It is much more quickly carried out than the method adopted under Portion A, and can be recommended as an easy means of separation of the mono- and di-phosphatides present in the ethereal extract of egg yolk, heart muscle, and probably in other substances.

In the following analyses Parts I and II were prepared as described under Portion A. Part III was prepared as mentioned under Portion B.

ANALYSIS

I

Nitrogen (Kjeldahl)

0·2596 gm. substance used 1·5 c.c. $\frac{n}{10}$ H_2SO_4 = 0·81 %
0·3122 " " " 1·82 c.c. " = 0·82 %

Phosphorus (Neumann)

0·2411 gm. substance used 15·66 c.c. $\frac{n}{2}$ NaOH = 3·60 %
0·1565 " " " 10·3 c.c. " = 3·64 %

Elementary Analysis

0·1625 gm. substance gave 0·3527 gm. CO_2 = 59·19 % C
and 0·1388 gm. H_2O = 9·56 % H

II

Nitrogen

0·3140 gm. substance used 1·78 c.c. $\frac{n}{10}$ H_2SO_4 = 0·794 %
0·6952 ,, ,, ,, 4 c.c. ,, = 0·806 %

Phosphorus

0·4139 gm. substance used 26·55 c.c. $\frac{n}{2}$ NaOH = 3·55 %
0·2729 ,, ,, ,, 17·56c.c. ,, = 3·56 %

Elementary Analysis

0·1541 gm. substance gave 0·3329 gm. CO_2 = 58·92 % C
and 0·1296 ,, H_2O = 9·41 % H
0·1701 ,, ,, ,, 0·3686 ,, CO_2 = 59·09 % C
and 0·1413 ,, H_2O = 9·29 % H
0·1448 ,, ,, ,, 0·3140 ,, CO_2 = 59·14 % C
and 0·1217 ,, H_2O = 9·40 % H

Iodine Number

0·1691 gm. substance bound 0·1317 gm. Iodine = 76·8
0·2254 ,, ,, ,, 0·1759 ,, ,, = 77·1

III

Nitrogen

0·4836 gm. substance used 2·85 c.c. $\frac{n}{10}$ H_2SO_4 = 0·83 %

Phosphorus

0·2132 gm. substance used 13·82 c.c. $\frac{n}{2}$ NaOH = 3·59 %

Elementary Analysis

0·1128 gm. substance gave 0·2453 gm. CO_2 = 59·31 % C
and 0·0963 ,, H_2O = 9·55 % H

	Portion I		Portion II			Portion III	Average	Average of Cuorin (Erlandsen)
	per cent.	per cent.	per cent.	per cent.	per cent.	per cent.		
C	59·19	—	58·92	59·09	59·14	59·31	59·12	61·63
H	9·56	—	9·41	9·29	9·4	9·55	9·44	9·03
N	0·81	0·82	—	0·794	0·806	0·83	0·812	1·015
P	3·60	3·64	—	3·55	3·56	3·59	3·59	4·46
O	—	—	—	—	—	—	27·048	—

Relation N : P = 1 : 2.

Since the only substance of this nature hitherto isolated is the cuorin of Erlandsen, a comparison of the two substances is of some interest. A reference to the analyses shows that they are not identical. It, for instance, we consider the percentage of N and P in the two compounds, it is seen that the difference is relatively high. Again, the O content of my substance is higher than that of cuorin. At first it was thought that this difference in percentage composition might possibly be dependent on the occurrence of a certain amount of oxidation, prior to, or during, the preparation of the substance, and the extreme facility with which cuorin undergoes oxidation lent colour to this view. An examination of the

figures, however, shows that possible oxidation does not account for the difference: *a priori* the probability of any marked oxidation was unlikely, since all manipulations were so conducted so as to exclude as far as possible the presence of oxygen.

Since cuorin contains a relatively higher percentage of N and P than this substance, it is obvious that the addition of more O would lower the N and P percentage, and so tend to lessen the difference in composition.

For a specimen of oxidised cuorin, Erlandsen gives the following figures: – O 30·72, N 0·92, P 4·06 per cent.; while my substance gives O 27, N 0·812, P 3·59 per cent.

If we consider cuorin oxidised only to the extent of 27 per cent. (instead of 30 per cent. as given), this portion must contain something over 0·92 per cent. N and something over 4·06 per cent. P. Since my substance, oxidized to 27 per cent., contains only 0·812 per cent. N and 3·59 per cent. P, it is clear that possible oxidation is not the cause of the difference. It is probable that this is really accounted for by a difference in the fatty acids of the two substances.

Again, though the substances differ somewhat in their iodine figures, this difference is not great enough to satisfy the assumption that the one is but an oxidized form of the other.

Whether the somewhat prolonged treatment with hot alcohol necessary for the isolation of the substance in a pure condition has any action in causing some decomposition, and so a slight change in the ultimate formula of the phosphatide is perhaps worth consideration; if this is so, it is possible that the exact percentage composition may vary slightly, depending on the amount of hot alcohol treatment. Since, however, the different portions mentioned above yielded practically the same results, this is not very probable; in any case, it does not affect the fact that in egg yolk there is contained a well defined lecithin-like substance containing N : P in the proportion of 1 : 2.

Properties

This substance is obtained as a yellowish brown brittle substance, which, after being carefully dried, is easily ground to an exceedingly fine powder; it is much more brittle than cuorin, and though hygroscopic, is by no means markedly so in comparison with certain other phosphatides. In common with all lecithins it undergoes oxidation on exposure to air. It is insoluble in cold alcohol but somewhat soluble in boiling alcohol, and thus differs from cuorin, which is said to be insoluble in boiling

alcohol; in chloroform, ether and petroleum ether it dissolves easily at the ordinary temperature. In ethyl acetate it dissolves on heating, to be thrown out on cooling. From its ethereal solution it is precipitated by acetone. With water it forms an emulsion. With platinum chloride it gives double salts, and by hydrolysis gives fatty acids and glycerophosphoric acid; no base of the nature of choline could be obtained. When heated to 90° to 100° C. it changes colour, but an exact melting point could not be determined. After hydrolysis with weak hydrochloric acid no reducing carbohydrate was obtained. All its general properties show that here we have a typical phosphatide substance.

White Substance Soluble in Hot Alcohol and Separating Out on Cooling

This substance (Portion *a*, page 169) was dissolved in hot alcohol, filtered hot, allowed to cool, and again filtered. By this means a voluminous precipitate of whitish snowflake-like material was obtained, which on drying shrunk to a very small volume. This process was repeated four times. By this means a fine whitish granular powder was obtained, which was completely soluble in ether, chloroform and benzene. On the addition of acetone to any of these solutions it was, after a little time, but not immediately, precipitated.

As some doubt exists as to the composition of this 'white substance,' Erlandsen having found N but no P in apparently similar material, while Thierfelder and Stern found both N and P, I made some further investigations with the following results.

In this particular preparation there was neither N nor P present. Melting point was easily observed being 62° to 63° C. When burned on platinum foil it left no inorganic residue. After boiling for some time with HCl no substances of a reducing nature were obtained. Saponification with $Ba(OH)_2$ failed to give any substances of basic nature precipitated by platinum chloride. From these observations it was apparent that this substance was probably of the nature of a fat. A combustion experiment showed this fat to be tripalmitin.

Analysis

0·1068 gm. substance gave 0·2964 gm. CO_2 = 75·70 % C
and 0·1179 ,, H_2O = 12·35 % H

Calculated for Tripalmitin

$C_{51}H_{98}O_6$ = 75·86 % C
and 12·24 % H

I would here remark that no investigation of any of the filtrates used in purifying the above substance was made. This tripalmitin seems to be somewhat soluble in 'lecithin,' so it is doubtful if it is possible to obtain 'lecithin' absolutely free from fat.

Summary

The ethereal extract of egg yolk contains as well as ordinary 'lecithin' with N : P as 1 : 1 another lecithin-like substance—a mon-amino-diphosphatide body—with N : P as 1 : 2.

This substance, as above obtained, differs somewhat in constitution and properties from the substance, cuorin, prepared from heart muscle, the difference being probably dependent on the presence of different fatty acids.

The other substance in the ethereal extract which dissolved in hot alcohol and separated out on cooling was found to be pure tripalmitin.

IODO-EOSIN AS A TEST FOR FREE ALKALI IN DRIED-UP PLANT TISSUES

By A. C. HOF, *Höchst a. Main.*

Communicated by Professor P. Ehrlich

(Received March 24th, 1909)

The free dye-acid of iodo-eosin has been employed by Professor Ehrlich as an extremely delicate reagent for free alkali in the red-blood corpuscles of man.[1]

The writer of the present note subsequently found that the same reaction proved also a useful test for the feeblest free alkalis in dried-up plant tissues.[2]

The dye used is iodo-eosin, the potassium salt of tetraiodo-fluoresceïn. Iodo-eosin as a salt dissolves in dilute alkalis with red colour, but is insoluble in ether or any other organic solvent. The free dye-acid, however, obtained as a yellow precipitate from the alkaline solution of iodo-eosin by adding hydrochloric acid in excess, dissolves easily in ether or in any other organic solvents, but is insoluble in water.

It is for this reason that the reaction can be made use of for testing free alkali in a perfectly dry tissue.

Suppose we have a free hand transverse section of a dry twig of a common forest-tree, for example, a spruce fir, and put the section into a solution of the dye-acid of iodo-eosin that has been dissolved in ether, leave it there for some minutes, then wash out the section thoroughly with ether. The preparation brought into xylene should now be examined under a low power of the microscope, when some of the histologically differentiated elements of the tissue will be stained distinctly red. In our section, for example, the cambium, the resin canals and the secreting cells lining the cavity appear plainly red-stained.

It is easy to see that these red-stained elements of the tissue must be those that contain free alkali, and, further, that their red colour is due to the red alkali salts formed.

We are justified in supposing that the free alkali located in these tissues is acting as a dye-base as soon as the dye-acid of iodo-eosin is

1. Ehrlich-Lazarus, *Die Anaemie*, Vienna, 1898.
2. A. C. Hof, *Botanisches Centralblatt*, LXXXIII, 1900.

added, yielding immediately the characteristic red dye-salt. If there is no water present the red colour remains in the tissue fixed at the exact place where it has been formed. Thus we get an exact idea of the distribution of free alkali in dry tissues.

Methods

Preparation of the dye-acid of iodo-eosin.—Dissolve 1 gramme iodo-eosin, the commercial dye, in 1 per cent. potassium hydroxide, add hydrochloric acid in excess. The dye-acid precipitates at once. Filter and wash the precipitate with hot water thoroughly till the filtrate is absolutely free from hydrochloric acid. Dry the precipitate of dye-acid and dissolve it in 100 c.cm. ether.

Staining of sections.—Bring the sections, either sectioned free hand or in a microtome, into the above solution of dye-acid, leave them in it for some minutes, wash out the sections carefully with ether, transfer to xylene, and seal preparation in Canada-balsam. For preserving the sections distinctly stained it is absolutely essential that the balsam used is of perfectly neutral reaction. The ordinary balsam bought from dealers in microscope supplies often reduces the dye-salts immediately. By this reduction of the dye the colourless compound—the leuco-base or leuco-compound—is formed.[3]

I venture to hope that the foregoing reaction, simple, effective and feasible as it is in the case of almost any drug, may be of some use to students of pharmacognosy.

3. P. G. Unna, *Centralblatt für Bakteriologie und Parasitenkunde*, Vol. III, 1888.

THE GROWTH OF THE *BACILLUS TUBERCULOSIS* AND OTHER MICRO-ORGANISMS IN DIFFERENT PERCENTAGES OF OXYGEN

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(*Received March 31st, 1909*)

The experiments here described were suggested by the seats of growth of the *Bacillus tuberculosis* in the body corresponding as they do to situations where there is a high pressure of carbon dioxide and a low pressure of oxygen. It was thought from this that the bacillus might either require a certain definite percentage of carbon dioxide in the air, or might be very sensitive to high pressures of oxygen, and only grow, as in the lungs or elsewhere, where the partial pressure of the oxygen is normally much lower than in the atmosphere.

The experiments did not quite justify the theory; but the theory served the most essential purpose of a theory in leading to experiment, and certain of the results have proved in some respects sufficiently interesting to warrant description.

These experiments are still being continued, but as far as they have gone their chief results may be briefly put as follows: The *Bacillus tuberculosis* either does not grow at all, or grows very badly in the entire absence of oxygen, or in presence of a partial pressure of oxygen amounting to 80 to 90 per cent. of an atmosphere.

A number of other organisms have also been tested, and certain of these, like the *Bacillus tuberculosis*, cease to grow in the higher oxygen percentages, while others appeared to be unaffected by the variations in oxygen. The experiments with the *Bacillus tuberculosis* will first be described, and afterwards those with the other organisms.

Experiment I.—Growth in large tubes with stopcocks, and analyses of the gases in the tubes after growth.

Since carbon dioxide is such a heavy gas, it was thought that under the ordinary condition of culture in test tubes with cotton-wool stoppers

there might be an accumulation of carbon dioxide in the culture tubes in which the bacilli might grow, as in the lung. Accordingly two extra large tubes of about 120 c.c. capacity were made, each with a draw-off tube for analysing the tube gases, closed by a small glass tap near the bottom; and one tube was plugged with cotton-wool in the usual way, while the other was closed above with a rubber cork, through which passed a small glass tube, also closed by a glass tap. Each tube was inoculated on a slant surface of glycerine agar[1] with a strain of Avian tubercle, and the two tubes were incubated alongside of each other for fourteen days at 36° C.

Then the gases in each tube were analysed by drawing samples off into a Hempel gas burette in the usual way. The tube plugged with cotton-wool showed 19·4 per cent. of oxygen and no carbon dioxide; while that closed air-tight by the rubber cork gave 18·7 per cent. of carbon dioxide and no oxygen. Hence the reply of the experiment is that there is no accumulation of carbon dioxide in growing in the ordinary way with wool-stoppered tubes. There was a good growth of the bacillus in both tubes. This appears to be contradictory to the result of some of the later experiments, which show no growth in hermetically sealed tubes in absence of oxygen; but it is to be remembered that in the later experiments ordinary small test tubes were used, in which the supply of oxygen would soon be exhausted, while here, in order to get sufficient gas samples, large tubes of about 120 c.c. capacity were used, and there would be a sufficient amount of oxygen to allow of a good growth before the oxygen became exhausted.

Experiment II.—Effect of high partial pressure of oxygen.

This experiment and some of the later ones were carried out in a small copper autoclave, kept in an incubator at 36 to 37° C., and filled with the desired oxygen mixture, while the controls were grown in the same incubator outside the autoclave but alongside it. The autoclave was used because it was intended to pass on later to oxygen pressures higher than atmospheric, but when it was found that growth was stopped at 80 to 90 per cent. of oxygen at ordinary atmospheric total pressure, the autoclave was not found to be necessary, and hence in later experiments a glass desiccator on a ground base with a mercury seal all round outside the glass junction was used, as subsequently described.

Six tubes containing cultures on glycerine agar of *Bacillus tuberculosis* (Avian) were used for the experiment, four being placed in the autoclave

1. Veal broth agar containing 5 %, glycerine, and 2 % peptone, and acidity $\frac{N}{100}$ to phenol phthalein.

with a vessel containing soda lime to absorb any carbon dioxide set free, while the other two were grown in air as controls outside the autoclave in the same incubator. The autoclave was exhausted and then joined up to a gasometer holding oxygen, prepared carefully in the laboratory from potassium permanganate.

The exhaustion and filling up was repeated four times, and at the end the autoclave atmosphere was found by analysis to contain 87·5 per cent. of oxygen. The experiment was begun on June 24th, 1908, and the autoclave was opened and results noted and compared with control on July 8th, 1908.

The following show the percentages of oxygen in the autoclave on different days: – June 24th, 87·5 per cent.; June 26th, 85·7 per cent.; July 1st, 85·1 per cent.; July 4th, 84 per cent.; July 8th, 63·2 per cent. There was always an analysis made for carbon dioxide, but none was found, showing that the soda lime was quite effective. The drop in oxygen in the last analysis indicates the starting of a very slow leak around the rim of the autoclave; but there was never less than sixty-three per cent. of oxygen, and in the earlier part the percentage was nearly ninety.

On opening the autoclave it was found that the four tubes grown inside showed practically no growth at all, while the two controls grown in air showed a moderate growth.

Experiment III. – Growth of Bacillus tuberculosis (Avian) in ordinary small test tubes (a) hermetically sealed, (b) stoppered with rubber corks, and (c) in controls grown in the ordinary fashion with cotton-wool stoppers.

Eight test tubes containing the glycerine agar culture medium were inoculated with Avian tubercle on June 17th, 1908; four of these were hermetically sealed off at the upper end, avoiding any injury to the medium; two were stoppered with ordinary rubber corks; and two were closed with cotton-wool in the usual fashion. The whole eight tubes were then incubated alongside of one another in the same incubator for twenty-one days (till July 8th, 1908), when the condition of growth was noted in each set, and the gases analysed in the hermetically sealed and in the rubber closed tubes respectively.

The two control tubes showed a good growth, the four hermetically sealed tubes showed very slight growth, and the two rubber stoppered tubes showed slightly more growth than the sealed tubes, but much less than the controls.

The glass point of one of the hermetically sealed tubes being broken under water, a negative pressure was shown by an in-rush of water. The

free capacity of the tube was about 40 c.c. and 26·8 c.c. of residual gas was obtained. This contained 0·7 c.c. of carbon dioxide (2·6 per cent.) and no measurable amount of oxygen, so that practically all the residual gas was nitrogen. A similar analysis in one of the rubber stoppered tubes gave 29·6 c.c. of total gas, containing 3 per cent. of carbon dioxide, no oxygen, and the balance nitrogen.

Experiment IV. Growth of Bacillus tuberculosis (Avian) in high partial pressure of oxygen.

Six tubes of glycerine agar, plugged in the usual fashion with cotton-wool and equally inoculated with Avian tubercle, were taken (July 15th, 1908), and of these four were grown in the autoclave in increased oxygen, while the other two were grown in air alongside in the same incubator. The autoclave after receiving the four tubes, and also an open vessel containing 25 grammes of soda lime, was screwed up, exhausted by a water pump, and allowed to suck in oxygen from a reservoir, the oxygen being made previously, as in all the experiments, from potassium permanganate. The exhaustion and filling was repeated five times, and the final percentage of oxygen in the autoclave was 82·6. The experiment was run for twenty-one days, viz., from July 15th to August 5th, 1908, and analyses of the autoclave atmosphere at intervals gave the following results:—July 18th, 75·5 per cent.; July 22nd, 70·9 per cent.; July 26th, 57·9 per cent.; August 5th, 50·3 per cent.

There is here again a slow leak in the autoclave packing, and an oxygen percentage varying from 82·6 at the beginning to 50·3 at the close. There was no appreciable amount of carbon dioxide present throughout.

Examined at the end the two control tubes show a fair growth, while the four tubes grown in the increased oxygen show practically no growth.

Experiment V. Growth in stoppered large tubes alongside cotton stoppered similar tubes.

In order to examine more fully the gaseous exchanges in growth in sealed tubes where the growth was finally inhibited, and also to examine the effect of the medium alone upon the air in the sealed tubes, the following experiments were carried out in larger tubes than usual, the capacity of each tube being about 120 c.c. Six culture tubes were taken for the experiment and treated as follows:—

A.—Control. Glycerine agar inoculated with Avian tubercle and plugged with cotton-wool in the usual manner.

B.—Same as A, but plugged air-tight with solid rubber stopper.

C.—Glycerine agar, *not inoculated*, but plugged exactly like B.

D and E.—Same as B, and inoculated, but with a glass tube sealed at outer end inserted through rubber cork so as to be easily broken afterwards in rubber tubing to allow sample of gases to be taken for analysis.

F.—Glycerine agar, *not inoculated*, arranged as in D and E.

The six tubes were grown together in the same incubator from July 23rd till August 21st, 1908 = twenty-nine days.

The final result was as follows:—

	Amount of Growth	*Analysis of Residual Gases*
A.	The whole surface of the medium covered with *T. B.* growth.	Open to air; therefore no analysis.
B.	Fair growth, roughly about one-fifth of surface covered	Total volume 83·4 c.c.; no oxygen; carbon dioxide 5·7 per cent.
C.	Not inoculated; no growth.	Total volume 84·6; 12·4 per cent. of oxygen; no carbon dioxide.
D.	Hardly any growth.	Total volume 74·8; no oxygen; 5·0 per cent. carbon dioxide.
E.	Moderate growth; less than B, more than D.	Total volume 82·9; no oxygen; 6·7 per cent. carbon dioxide.
F.	No inoculation; no growth.	Total volume 80; 11·5 per cent. oxygen; no carbon dioxide.

This experiment shows that the control grown under atmospheric oxygen gives by far the best growth; that there is an up-take of oxygen by the medium alone but no output of carbon dioxide, about half the available oxygen being so used up in the time; and that in the inoculated and stoppered tubes all the oxygen disappears, but only about one-third of the corresponding amount of carbon dioxide appears.

Experiment VI. Growth of strains of Bacillus tuberculosis of Avian, Bovine and Human origin, in increased percentage of oxygen.

This experiment, instead of being carried out in the autoclave, was done in a large desiccator or bell-jar, resting on a well-fitting ground glass base, and having a glass tube and well-fitting tap above fixed in a rubber cork in a wide tubulure. In order to keep a quite tight joint below, a mercury seal was arranged all round the base by constructing a circular mound of putty all round about a centimetre high and of about two centimetres greater diameter than the rim of the bell-jar. In this way the slow leak of the autoclave experiments was avoided, and, as shown by the analyses in this and and the succeeding experiments, the percentage of oxygen was kept high and very nearly constant throughout the necessarily somewhat prolonged experiments.

Two tubes each were inoculated on glycerine agar with tubercle bacilli of avian, bovine and human origin, and plugged in the ordinary way with cotton-wool. These were placed in the bell-jar above described along with 20 grammes of soda lime in an open flat vessel. One tube of each strain was inoculated and grown in air outside the bell-jar, but close alongside it in the same incubator, to serve as a control.

The experiment was commenced on the 2nd December, 1908, and concluded on the 24th December, 1908 = twenty-two days. The percentage of oxygen was got up at the commencement in the usual way by exhausting and connecting with a reservoir containing permanganate oxygen. The initial value for the oxygen percentage was 77·3 on December 2nd and 76·3 on December 24th, showing that there was no appreciable leak.

Examination of the nine tubes at the later date shows in every case that the growth is stronger and thicker in the controls than in those grown in the oxygen. This is particularly well shown in the human strain, in which one of the two tubes shows hardly any growth and the other a very scanty growth, while there is a much better growth in the control.

The same holds for the bovine growths, but all three tubes are somewhat further advanced.

In the avian the growth is considerable in both oxygen grown tubes and controls, but the control is thicker and better grown.

Experiment VII. Growth of Bacillus tuberculosis (Human) in increased oxygen percentage.

Three tubes were used of human strain, grown as before on glycerine agar. Two were grown in the bell-jar with increased oxygen, one as control in air outside and close to the bell-jar in the same incubator. Bell-jar exhausted and refilled six times; final oxygen percentage on January 1st, 1909, was 90·5. Experiment continued till January 27th = twenty-six days, when percentage of oxygen was 88·9; no carbon dioxide present.

Examination showed that there had been no growth in the two tubes kept in the oxygen, and a moderate but quite obvious growth on the air grown control tube. As the control had dried somewhat during the twenty-six days of the experiment, in the succeeding experiment this was obviated by placing the control also under a similar bell-jar and in all respects in similar condition to the other set of tubes, except that the growth was made in air instead of in a high percentage of oxygen. But

it may be pointed out in regard to this present experiment, that any drying would militate against growth rather than favour it, yet the control had obviously grown, while the oxygen grown tubes had not grown.

Experiment VIII.—Growth of Bacillus tuberculosis (Human and Bovine) in increased oxygen percentage.

Four culture tubes each, of human and of bovine strains were made, and wool-stoppered in the usual manner.

Two tubes of each strain were placed in the oxygen bell-jar, which was exhausted and refilled in the usual way five times, the final percentage of oxygen obtained being 87·6. Alongside this was placed another similar bell-jar, fitted up in the same way but filled with atmospheric air, and in this likewise two tubes of each strain were placed.

The experiment was commenced on February 14th, 1909, and on February 24th and from that date onward a most marked and increasing difference was observable in the two sets of tubes; those in the oxygen had not appreciably grown, while the surfaces of the air cultures were dotted over with vigorous colonies of growth. The experiment was discontinued on March 3rd, when the percentage of oxygen was found to be 75.

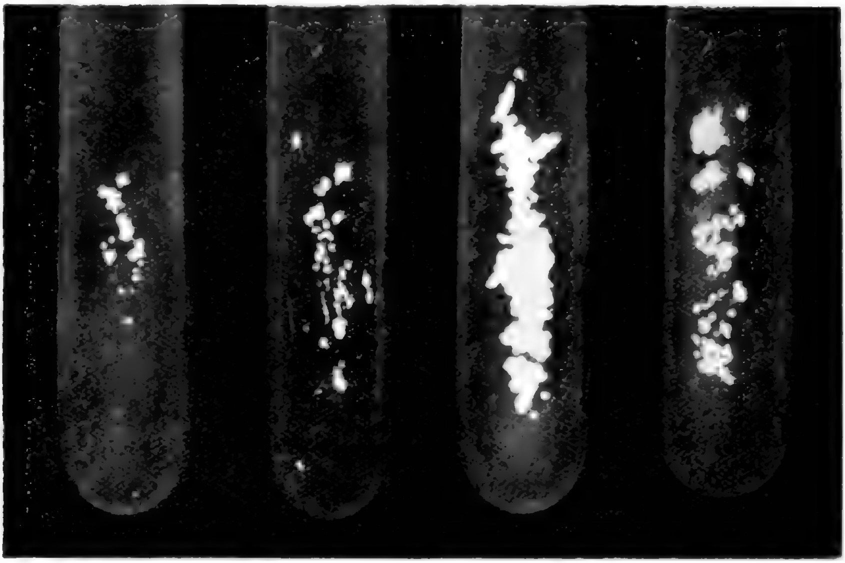
The photographs shown in fig. 1 were made from this experiment.

In the photograph the four upper tubes were those grown in the oxygen, and the four lower tubes were those grown in air. In each series the two tubes on the left-hand side are bovine tubercle, and the two tubes on the right are human tubercle.

In order to test whether the growth of the bacillus was only inhibited by the oxygen, the organisms still remaining alive, or whether they had been permanently destroyed by their stay in the higher oxygen percentage, sub-cultures were made of both bovine and human, from both the tubes in air and those in oxygen. The results showed a good growth in about fourteen days on the sub-cultures from the air grown tubes, while the sub-cultures from the previously oxygen grown tubes showed no growth, but turned brown where there were specks of the inoculated material on the agar. These sub-cultures were, of course, in both cases grown in air. The experiment, therefore, shows that in this case the oxygen had killed the bacteria; but the experiment requires repetition.

Experiment IX.—Growth of Bacillus tuberculosis (Human and Bovine) in increased oxygen percentage.

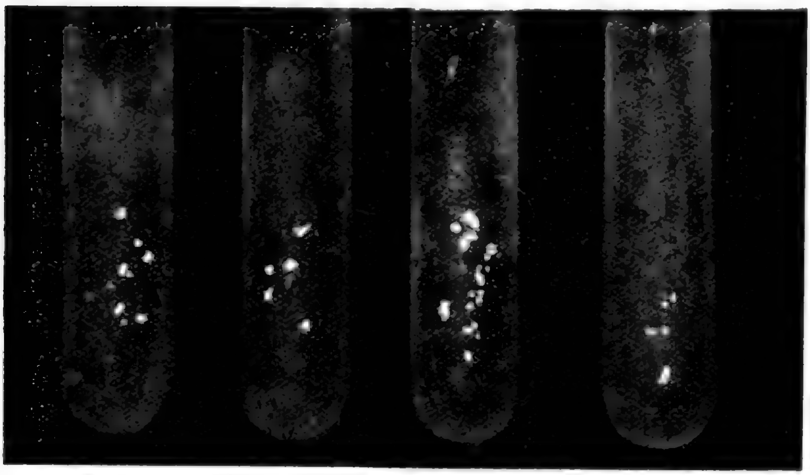
In this experiment other micro-organisms were cultured alongside the *B. tuberculosis* under the same oxygen and air bell-jars, but the



1 2 3 4

1. *Bacillus tuberculosis* (Bovine). 3. *Bacillus tuberculosis* (Human).
2. *Bacillus tuberculosis* (Bovine). 4. *Bacillus tuberculosis* (Human).

GROWN IN AIR.



1 2. 3. 4.

1. *Bacillus tuberculosis* (Bovine). 3. *Bacillus tuberculosis* (Human).
2. *Bacillus tuberculosis* (Bovine). 4. *Bacillus tuberculosis* (Human).

GROWN IN OXYGEN.

FIG. 1. TUBERCLE BACILLUS GROWN IN AIR AND IN OXYGEN.

growth of the tubercle bacillus is so slow that the tubes containing the tubercle bacilli had to be kept in the bell-jar during more than one experiment on the other organisms, which grew abundantly in two or three days. For clearness of description the effects on the tubercle bacilli are described separately, but the opening of the desiccator to remove and replace other organisms is noted, and the oxygen percentage at each period in the experiment was usually determined each time the atmosphere was changed.

March 5th, 1909. Two tubes of *Bacillus tuberculosis* (human) and two of *B. tuberculosis* (bovine), which had been inoculated March 2nd, were placed in the oxygen bell-jar containing 90·0 per cent. of oxygen.

Four similar tubes, two of each strain, inoculated at the same time and grown in the interval alongside in the same incubator, were placed in the similar control bell-jar in atmospheric air.

The two sets of tubes show a commencing growth in each case, but slightly advanced.

The two bell-jars placed alongside of each other in the incubator at 6-30 p.m., March 5th, the eight tubes being very similar as to growth.

The two bell-jars were opened on March 9th to remove the tubes with other micro-organisms described in the next experiment (see Expt. X). The growths of the tubercle bacillus had not yet advanced far enough for comparison, so they were replaced and the oxygen bell-jar again charged by six exhaustions and refilling with oxygen from the reservoir.[2] The oxygen percentage at this second charging was 91·7 per cent., and the tubes were not again disturbed till March 11th, when they were reopened to remove the other organisms grown in tubes alongside. The tubes were again replaced in their respective bell-jars, and the oxygen percentage raised in the oxygen bell-jar to 88·8.

The bell-jars were again opened temporarily and at once restarted on March 12th, March 16th, and finally for examination at the conclusion of the experiment on March 17th, after twelve days' growth in the oxygen, and in the air alongside for the controls.

The oxygen percentage on March 16th was 90·8 per cent.; it was not determined on March 12th or 17th.

All four tubes grown in air showed good growths at the end of the

2. That the failure of the Bacilli to grow was not due to the frequent exhausting of the bell-jar, is shown by the vigorous growth under such conditions of the *B. coli* and *B. typhosus* grown alongside, and also in subsequent experiments.

period; the human and bovine strains in this experiment grew at about the same rate.

None of the four tubes grown in oxygen showed any growth whatever, and the small amount of growth which had occurred in air during the period from March 2nd till March 5th, when they were placed in the higher oxygen percentage, had turned dark brown in colour, the cultures being obviously dead.

Experiment X. Growth of other organisms—Staphylococcus albus, and Bacillus coli—in increased oxygen percentage.

March 5th, at 6-30 p.m. Two tubes of *Staph. albus* and two tubes of *B. coli* immediately after inoculation were placed in the oxygen bell-jar and grown in 90 per cent. oxygen. As controls, one tube of each organism was grown in the air bell-jar alongside.

March 9th, 2-30 p.m. The two bell-jars opened and growths compared.

The two tubes of *B. coli* grown in the oxygen showed a growth quite equal to that of the tube grown in air. (See fig. 2.)

On the other hand neither of the two tubes of *Staph. albus* grown in the oxygen showed any appreciable growth, while the control tube grown in air had a very good growth, the whole surface of the medium being covered. (See fig. 3.)

The six tubes were at once photographed, and the results are shown in figs. 2 and 3.

Experiment XI.—Growth in increased oxygen percentage of Staphylococcus aureus, Bacillus coli, and Bacillus typhosus.

March 9th till March 11th, 1909. Two tubes of each organism were grown in the oxygen and in the air bell-jars respectively. Growth was commenced on afternoon of March 9th. When examined without opening on March 10th, afternoon, all six control tubes in *air* show good growths; in the *oxygen* the *B. coli* and *B. typhosus* show good growths, while the *Staph. aureus* shows no growth at all.

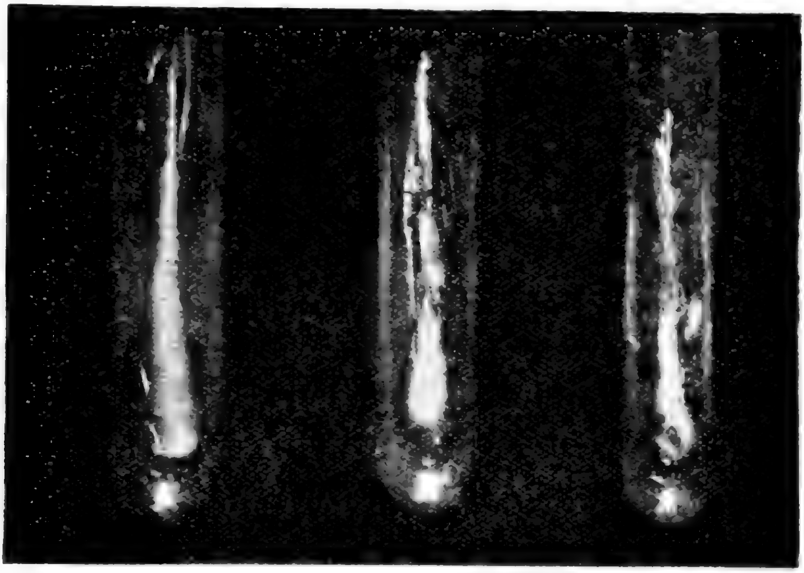
March 11th. Both bell-jars opened and tubes examined.

All four *B. coli* tubes show full normal growth.

All four *B. typhosus* tubes show full normal growth.

The two *Staph. aureus* grown in the *air* bell-jar showed full normal growth; but the two grown in oxygen showed the merest trace of growth.

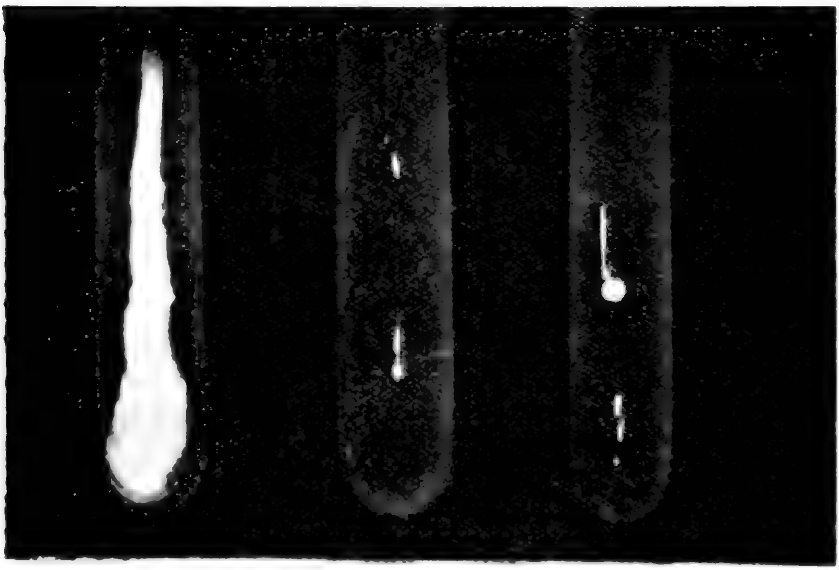
Experiment XII.—Growth in increased oxygen percentage of Bacillus pyocyaneus, Vibrio cholerae, Bacillus dysenteriae (Shiga), Bacillus dysenteriae (Flexner), and Staphylococcus citreus.



1. 2. 3.

1. *Bacillus coli* grown in air.
2. *Bacillus coli* grown in oxygen.
3. *Bacillus coli* grown in oxygen.

FIG. 2.—GROWTH OF COLON BACILLUS IN AIR AND IN OXYGEN



1. [illegible]
2. *Staphylococcus aureus* grown in oxygen.
3. *Staphylococcus aureus* grown in oxygen.

FIG. 3.—GROWTH OF STAPHYLOCOCCUS AUREUS IN AIR AND IN OXYGEN

Experiment commenced at 4 p.m., March 11th; growths examined March 12th at 4 p.m.; oxygen percentage = 88·8. Two tubes of each organism grown in air and two in oxygen.

All ten tubes grown in air show a good full growth. Of the tubes grown in oxygen—

Vibrio cholerae shows good growth, as good as control.

B. dysenteriae (Flexner), growth as good as control.

B. pyocyaneus, growth as good as control.

B. dysenteriae (Shiga) shows no growth. (See, however, Expt. XV.)

Staph. citreus. Both the oxygen tubes show an appreciable growth, but much less than controls.

Experiment XIII.—Growth in increased oxygen percentage of Bacillus diphtheriae, Staphylococcus citreus, Staphylococcus aureus, Staphylococcus albus, Bacillus dysenteriae (Shiga), and Bacillus dysenteriae (Flexner).

Examined after twenty-four hours, the two *Staph. citreus* tubes grown in oxygen show a quite perceptible growth, rather more than either *Staph. albus* or *Staph. aureus*, but nothing like the growth of the controls grown in air. The two tubes of *Staph. albus* and *Staph. aureus* respectively from oxygen bell-jar show just the merest trace of growth, practically no growth, while the four air grown tubes show a good growth.

The two *B. dysenteriae* (Shiga) tubes from the oxygen show no growth at all; the two from the air are well grown (see Expt. XV). All four from *B. dysenteriae* (Flexner) tubes, on the other hand, show good growths both in oxygen and in air, there being no appreciable difference.

None of the four *B. diptheriae* tubes show a good growth, but the oxygen tubes appear to be a little less than the air grown tubes.

Experiment XIV.—Growth in increased oxygen percentage of Staphylococcus albus, Staphylococcus aureus, Staphylococcus citreus, Bacillus dysenteriae (Shiga), and Bacillus dysenteriae (Kruse).

Four tubes of each of the above organisms were cultivated from March 19th (afternoon) till March 22nd (afternoon). Two of each in air and two in oxygen of 90 per cent.

All the tubes grown in the air showed good growths.

Grown in the oxygen. *B. coli*, good growth, quite as good as controls. *Staph. albus*, fair growth, but not nearly so good as controls. *Staph. aureus*, poor growth, less than *Staph. albus* and not nearly so good as

controls. *Staph. citreus*, fairly good growth, much better than the two previous, and approximately half its own controls. *B. typhosus*, a very good growth, perhaps just a little poorer than the control. *B. dysenteriae* (Shiga), a very poor growth, not to be compared to the control. *B. dysenteriae* (Kruse), poor growth, less than one-third that of control.

In this experiment, which was of longer duration than the previous ones with the more rapidly growing organisms, there was distinctly more growth in the oxygen grown cultures than in the shorter experiments, but, at the same time, the inhibition of certain of the organisms was undoubted. It was also particularly noticeable here, as was more or less obvious throughout the whole series of experiments, that in those cases where the bacteria grew in a suppressed fashion, under the inhibition of the oxygen, that the cultures, instead of forming a more or less uniform mass or smear over the surface of the culture medium, consisted of a number of very marked round colonies heaped up.

In several cases where tubes of staphylococcus which had not appreciably grown while in the oxygen had been left on longer *in air* in the incubator, it was noticed that these recovered and grew fairly well in a rather spotted and heaped up fashion. So that the shorter stay in oxygen does not appear to kill the more rapidly growing organisms in the same manner as the prolonged stay in oxygen appears to kill the tubercle bacillus.

To test the effects of a more prolonged stay in oxygen Experiment XV was carried out.

Experiment XV.—More prolonged growth in increased oxygen percentage of Bacillus coli, Staphylococcus citreus, Staphylococcus aureus, Staphylococcus albus, Bacillus diphtheriae, Bacillus typhosus, Bacillus dysenteriae (Flexner), Bacillus dysenteriae (Kruse), and Bacillus dysenteriae (Shiga).

Two tubes of each of these organisms were grown in oxygen of 90·5 per cent. and in air respectively, the experiment being continued from the afternoon of March 25th to that of March 30th.

The following is the comparison of the two sets of growths:—

B. coli, typhosus, diphtheriae show no difference in air or oxygen of any great magnitude, the air tubes perhaps slightly better grown, but difference very slight.

Staph. aureus shows a good growth in air, none in oxygen.

Staph. citreus shows a good growth in air and a considerable growth in oxygen, but in round heaped up masses not occupying the whole surface.

Staph. albus shows a good growth in air, but practically none in oxygen.

B. dysenteriae (Shiga) shows growth in both, but distinctly less in oxygen.

B. dysenteriae (Flexner) shows growth in both, somewhat less in oxygen.

B. dysenteriae (Kruse) was nearly equally grown in both sets of tubes.

We desire to express our thanks to Mr. Arthur Webster for much valuable assistance in the experimental work and analyses.

THE ELECTRICAL FORCES OF MITOSIS AND THE ORIGIN OF CANCER

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Professor Hartog has recently brought forward the interesting suggestion that the phenomena of mitosis, that is to say the well-known mitotic figures, are due to the existence of a dual force, as for example, a magnetic or, better, electrical type. Without pre-judging its nature, he calls this force mitokinetism. He introduces the conception of relative permeability in elucidating the behaviour of this dual force in comparison with the phenomena of magnetism. He goes on to say that as the cell structures are all material the conception of geometrical lines of force is adequate to explain them. He says that the effect of stresses within a mixture of substances which are of different permeability and free to arrange themselves will be to segregate out the more permeable material in strands along the lines of force. While the idea of an opposite polarity is reasonable, it is difficult to accept the polarity as being magnetic in any way, because there does not seem to be present any mechanism whereby magnetic stresses are to be produced. On the other hand, the substitution of an electrostatic difference of potential for the magnetic removes these difficulties, for it would appear that in the configuration of the protein material we have at hand all the necessary conditions for the establishment of such charges. The - NH - CO - linking which, from a physico-chemical point of view, is both acid and basic in character, or, as usually called, amphoteric, possesses residual affinity of two opposite types, and it is in the existence of these two types that we can obtain the mechanism for the establishment of electrostatic difference of potential. Although in the cytoplasm and centrosome we can find an analogy with the solvent and the dissolved and ionised salt in inorganic chemistry, yet it must be remembered that in the organic cell the phenomena must be those of colloids, and for this reason we are somewhat hampered by ignorance of the nature of colloidal substances. It is possible, however, to develop a theory that electrically charged colloids play a very important rôle in mitosis, a theory which leads to some very interesting results. When a crystalline salt, as, for example, sodium chloride, is dissolved in

water the residual affinity of the water molecules causes them to condense round and form loose hydrate compounds with one or both of the sodium and chlorine portions. The lines of force due to the chemical combination of the sodium and chlorine are thereby weakened, and by virtue of their velocity of movement by diffusion the two ions get separated, becoming at the same time seats of positive and negative charges respectively. In an analogous fashion the cytoplasm can resolve a discrete molecule or complex of molecules of amphoteric type into two oppositely charged portions. The cytoplasmic mass can, by virtue of its two types of residual affinity, form loose compounds with the two portions of the simpler compound. The lines of force between the two will be weakened, and the two portions can be separated and become seats of positive and negative charges respectively. It appears, therefore, a justifiable assumption that electrostatic differences of potential are established during mitosis and that the two centrosomes represent the location of two of these charges. Very much the same reasoning may be applied to the chromosomes or chromatin granules; these, by the same process as detailed above, can become resolved into two oppositely charged portions. Without in any way assuming that these differences of potential cause the phenomena of mitosis to occur, it is very difficult to believe that they are not produced when mitosis does occur. How far they act as the *causae causantes* is not determinable with any certainty in the present state of our knowledge of the vital processes, but our knowledge of the chemical and physical properties of the protein configuration leads us to the standpoint that the resolution of the centrosomes and chromatin granules must be accompanied by the establishment of definite electrostatic differences of potential.

If now we consider the probable influence of the lines of force between two oppositely charged bodies upon a colloidal mass it will be seen that the colloid will tend to coagulate. The well-known coagulation of colloids in the presence of an ionised salt is now attributed to the alteration in their surface tension by the lines of force between the ions passing through the surface. If the view be accepted that a colloidal solution is due to the existence of a negative surface tension, that is to say, a tendency to form as great a surface as possible, the penetration of that surface by the lines of force between the ions becomes at once comprehensible when a colloidal and ionised solution are mixed together. Applying this view to the prophase of mitosis, the penetration of the nucleus by the lines of force between the centrosomes will cause the coagulation or partial coagulation of the chromatin in the chromosomes,

a coagulation which will increase, the greater the number the lines of force which pass through the nucleus. This perhaps gives a reason for the condensation of the reticular structure in the chromosomes in the earliest prophase of mitosis.

To deal next with the chromosomes themselves. There is little doubt that these consist of discrete particles or granules of chromatin, and, in all probability, each of these is resolved into two daughter particles charged with positive and negative electricity respectively. The chromosome, by virtue of the splitting of each of its particles into two, forms two daughter chromosomes, one charged with negative and the other with positive electricity. It may be argued here that the splitting of the chromosomes occurs at a much later stage than the commencement of separation of the centrosomes. This, however, would be the result of the fact that during the earlier phases the chromosomes are coagulating; for it is not likely that the resolution of the material can occur until the coagulation of the granule into a discrete particle with a definite surface has taken place.

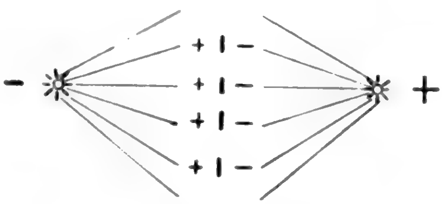


FIG. 1.

The first stage of somatic mitosis would, therefore, appear to be the resolution and separation of the centrosomes with their definite, equal and opposite charges of electricity. The lines of force between the two centrosomes as these lines penetrate the nucleus cause the coagulation or condensation of the chromosomes. The next stage is the resolution of the chromatin granules, each into two daughter particles of equal and opposite charge, and this is eventually followed by the splitting of the chromosomes. The first evidence of an incipient splitting of the chromosomes will be their polarisation; that is to say, each chromosome will be half positively and half negatively electrified and will tend, therefore, to move and take up its position in the equatorial plane of the mitotic figure (as in fig. 1).

The next change in the mitosis will be the actual parting of the

chromosomes into two daughter chromosomes, which by reason of the electrostatic attraction will migrate to the oppositely charged centrosome. When the daughter chromosomes arrive at the centrosomes the electrical charges will be neutralised. It is necessary to assume that the charges are entirely neutralised, for if there remain a balance of positive or negative electricity, this will tend to mount up in successive divisions—a condition which it is quite impossible to accept. We are, therefore, bound to take the view that the sum of all the charges on one set of daughter chromosomes is equal and opposite to that upon one centrosome, and that perfect neutrality of charge is established at the end of each somatic mitosis. When the lines of force cease to exist, each chromosome tends to become again de-coagulated. It at once begins to increase its surface, which it does by means of growing processes which extend until the whole nucleus appears to all intents and purposes structureless. The chromosomes, however, must preserve their individuality, although their processes appear inextricably intermingled. The application of the lines of force due to the commencement of a new mitosis will at once cause each chromosome to contract and condense until it exists once again as a dense individual, capable of being highly stained.

We have not considered as yet several of the attendant phenomena of mitosis: for example, no reason has been advanced for the separation of the centrosomes, the formation of the spindle figure, and also the disappearance and the reappearance of the nuclear wall.

Considering first the separation of the centrosomes, which appears to us to be the most important feature of mitosis, it must be remembered that in the analogous case of ion formation in aqueous solution the separation of the ions is due to diffusion. But this is quite inapplicable to the present case. It is equally necessary, however, to postulate some definite influence which separates the centrosomes—an influence which is stronger than and quite apart from the electrical forces: for these would naturally tend to draw the two centrosomes together. In other words, the electrical force cannot be the *causa causans* of mitosis, but must be a concomitant phenomenon. This fact cannot be too strongly insisted upon, for although the conditions of a living cell would seem to be such that the vital units must become resolved into two oppositely charged masses, yet unless some definite and separate stress were present, the two oppositely charged masses would lie side by side without any electrical influence on their surroundings. As stated above, the resolution into oppositely charged masses is the natural result of loose combination between the vital unit

and surrounding medium, and, again, these loose combinations are themselves the result of the chemical structure of the cell materials. These loose compounds must play a very important part in the general life of the cell, as, for example, in the growth of the chromatin. There is no doubt a strong separative force at work—a force which would appear to be connected with the mass division of the cytoplasm. It would seem that this mass division is an inherent vital property of living cytoplasm; and we incline to the view that it is the real *causa causans* of mitosis, and that the phenomena described above are produced by the mass division taking place. There is a sound foundation for our view, for it has been shown that the periodic activity of cytoplasm is independent of both nucleus and centrosome. For example, in the case of a fertilized egg divided into two portions, one of which contains the nucleus and the other not, the behaviour of the enucleated portion is most remarkable. It forms three times in succession a polar lobe at the same time that the nuclear half is dividing, becoming spherical after each period without dividing. At the fourth cleavage a fourth lobe is formed, which is not re-absorbed but grows steadily larger, so that the fragment appears finally to be divided into two. The activity of the enucleated half is thus not merely rhythmic in character but changes in character at the fourth cleavage when in normal development the polar lobe no longer forms a temporary structure but is permanently cut off by cell division. The cytoplasm, therefore, would seem to possess a power of mass division a power which is also periodic in its action, its periodicity no doubt depending upon the cytoplasm reaching a certain stage of development during the vegetative period of the cell. The existence of this power of mass division possessed by the cytoplasm gives a reasonable explanation for the separation of the centrosomes. When the period has been reached, the first feature of the phenomenon is the resolution of the centrosome from the previous division into two oppositely charged centrosomes. When the mass division begins the axis is determined by the position of the centrosomes, so that they are drawn apart. The electrical forces brought into play cause the condensation or coagulation of the chromosomes, their resolution and migration, and, finally, when the mass division of the cytoplasm is finished the formation of two daughter cells. By the statement that the axis of mass division is determined by the centrosomes we mean that the two daughter centrosomes are separated, because one must be in each daughter mass of cytoplasm. This fact cannot be a matter of chance, for if it were so, many more divisions of cytoplasm

would occur than mitoses of the nucleus, which is absurd. It is a natural sequence that the line of cleavage of the cytoplasm is determined by the centrosomes. This would seem to be the normal order of events in any mitosis, but there is no reason why certain minor details should not be altered either as regards their character or their position in the scale of operations. Such variations need not, and do not, militate against the electrical theory in any way. For example, in many cases the centrosome in the daughter cell divides immediately after the mitosis is finished in readiness for the next division. All that the theory demands is that the centrosome divide into two; the period at which this occurs is not of any moment, but the fact that is of the greatest importance is that the two new centrosomes never get separated except during mitosis, and then only with the formation of the spindle figure.

In reference to the statement that the axis of the mass division is determined by the centrosomes, O. and R. Hertwig[1] and also Roux[2] have noticed that as a rule the plane of division of a non-spherical cell is at right angles with the direction of the greatest diameter or extension, and Driesch has shown that if the newly fertilized egg of the sea urchin be gently pressed under a cover glass, so that it is slightly flattened, the plane of division is at right angles to the slide. The position of the plane of cleavage is determined by the position of the nuclear spindle, and this depends upon the position of the centrosomes. Moreover, in the process of cell division the egg of some animals becomes elliptic with the long axis falling in the direction of the common diameter of the amphiaster. This has given rise to the idea that it is the spindle itself which elongates the egg, but Loeb[3] has often noticed that the elongation, though in the direction of the spindle, always occurs immediately before the cell division.

Again, the possession by the cytoplasm of a power of mass division will give an explanation of the phenomenon of streaming which is observed during mitosis, for the streaming will merely be the flowing of the cytoplasm from the cleavage plane into two daughter masses. Inasmuch as the sum of the masses of the cytoplasm of the two daughter cells is less than that of the mother cell, it at once suggests itself that the mass division is caused in the first place by a loss of water from the periphery of the cell by osmosis.

1. *Untersuch. zur Morphol. und Physiol. der Zelle*, V, 1887.
2. *Breslauer Arzt. Zeit.*, 1885.
3. Loeb, *Dynamics of Living Matter*, p. 64. Columbia University Press, 1906.

To give an explanation of the formation of the asters and spindle figure of mitosis is not easy. The natural view to take would be that these are due to the coagulation or condensation of the cytoplasm along paths parallel to the lines of force, using the same argument as in the case of the chromosomes above. Furthermore, it is quite true that in many cases the mitotic diagrams present great similarity with the lines of force between oppositely charged bodies. This view has been previously advanced by A. Fischer.[1] In certain cases, however, the rays from the two asters appear to cross one another—an effect which is impossible if they are simply due to lines of force. But it may be that this is only an apparent effect due to the point of view of the phenomenon. If this be a real crossing it is clear that the rays cannot be due to threads of coagulated cytoplasm; although it might be possible to look upon them as direct growths from the centrosomes, yet the former explanation would seem far more reasonable, provided that the difficulty as regards their crossing one another be surmounted. Of course it must not be forgotten that the separation of the centrosomes by the mass division of the cytoplasm will induce stresses which may disturb the position of the threads. On the whole the evidence would appear to support the view that the mitotic figures are due to the coagulation of the cytoplasm under the influence of the lines of force.

In the present state of our knowledge of the chemistry of cell physiology it is impossible to account for the disappearance of the nuclear membrane during mitosis and its reappearance after the process is finished. We might say that the membrane is due to a definite chemical reaction between the nucleic acid and the cytoplasm, and that this reaction is reversed under the stimulus of the lines of force, so that the membrane disappears only to reappear when the force lines die away; but this can only be pure hypothesis.

We may next turn our attention to the maturation divisions of the germ cells, and investigate the relations which exist between the electric charges in these cases. The results obtained are peculiarly interesting, inasmuch as it seems absolutely certain that complete electrical neutrality does not, and cannot, result from these divisions.

The first fact which we are met with in these divisions is the fusion together of the chromosomes in pairs to give the meiotic gemini. In order to account for this and bring it into line with the other phenomena it is necessary to assume the existence of some form of polarity difference

1. *Fixierung, Farbung und Bau des Protoplasmas*, 1899.

between each of the two individual chromosomes, which fuse together to form the gemini. All recent cytological research leads to the view that in each case it is one paternal and one maternal chromosome which fuse together, producing a bi-polar twin; and, moreover, that it is not any chance pair which fuse together, but there exists some type of selective pairing between paternal and maternal chromosomes. For this pairing there must exist in the cell some opposite polarity between the two members of each twin a polarity which may well be of electrical type. This would point to the existence of some difference in electrical charge between the paternal and maternal chromosomes which comes into play during the long resting period of the germ cell. That an electrical attraction can be produced between paternal and maternal chromatin in the germ cell follows readily from the general theory, but its consideration may be postponed for the moment. It must be confessed that the phases of the phenomena of maturation divisions differ so much according to various observers that it is impossible to deal with more than what appear to be the most typical cases; and we must content ourselves with pointing out how the general relation between the charges is not altered in any of the cases observed.

The simplest case to deal with is when no tetrads are formed and when the first maturation division takes place between the split halves of the meiotic gemini, while the second division is a somatic division of the reduced number of chromosomes. It is necessary, in order to follow out the distribution of the charges in the maturation divisions, that the relative values of the charges upon the centrosome and chromosome be considered. Beyond the bare statement that it is essential that at the end of each somatic mitosis the charges upon the centrosomes must be neutralised by the chromosomes nothing has been stated as to the relative values of the charges. The establishment of electrical neutrality at the close of each somatic mitosis is of a very great importance, for if by some means or other the charges were not neutralised entirely and a small amount were left over in the daughter cells, subsequently this would go on mounting up steadily in successive divisions with no apparent limit. This is, of course, impossible of acceptance, and therefore we are driven to the conclusion, which is indeed the simplest, that complete neutrality obtains at the end of every somatic mitosis. We must therefore equate the charge upon each centrosome to the sum of the charges upon the daughter chromosomes, and in doing this it will be convenient to speak of the charge upon each centrosome as a unit-charge of positive or

negative electricity respectively. In the case, for example, of an individual with four somatic chromosomes, the sum of the charges upon the four daughter chromosomes which migrate to one daughter cell must equal the unit charge upon the corresponding centrosome. We therefore in this would expect the average charge upon each of the chromosomes to be one quarter of the unit charge, but it may be pointed out that there is no *à priori* reason why the charges on all the chromosomes should be equal in amount, the essential condition only being that the sum of the charges on them be equal to the unit charge.

Turning now to the germ cells we may say, as above, that a definite difference of potential is developed between paternal and maternal chromosomes; and let us say, merely for purposes of argument, that this difference of potential is half that carried by the chromosomes in somatic mitosis. If we continue to deal with the case of an individual with four somatic chromosomes, then we will assume the difference between paternal and maternal chromosomes in the germ cells as one-eighth of the unit-charge. This difference of potential will cause the fusion of the paternal and maternal chromosomes in pairs, which will be polarised, and carry at each end a one-eighth unit-charge of positive and negative electricity respectively. The result will be that the gemini will carry themselves across the equatorial plane of the spindle figure, with their ends turned towards the centrosomes, as in fig. 2.

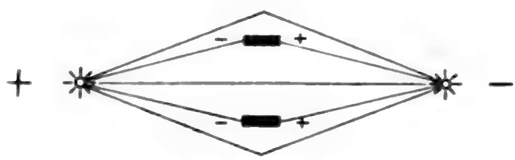


FIG. 2.

When the gemini split again, and the two chromosomes migrate to the centrosomes, the charges will not be neutralised, but in each daughter cell there will be left a residue of electricity. When the meiotic gemini split they each give two halves carrying an eighth positive and negative charge respectively, and in fig. 2 the two positive halves migrate to the centrosome on the right and the two negative ones migrate to the centrosome on the left. When the two positive halves arrive at the negative centrosome they each bring a one-eighth unit, that is to say, one-quarter unit positive electricity altogether. Since the centrosome

carries a whole unit of negative electricity, we have one unit negative and one-quarter unit of positive electricity, which leaves a balance of three-quarter positive electricity. Similarly at the other side there will be left a residue of three-quarter unit negative electricity. On these lines it is clearly impossible for the charges to be neutralised in the daughter cells, for the only condition under which this could be secured is that the difference of potential between the paternal and maternal chromosomes was half a unit-charge, or twice as much as between the daughter chromosomes in the previous somatic mitoses. Such a large difference of potential is out of the question, for it would be impossible for such a charge to lie dormant through the various somatic divisions which occurred previously to the maturation divisions. We must conclude, therefore, that whatever may be the real value of the potential difference between paternal and maternal chromosomes, a residual charge is left in the daughter cells of the first maturation division, and at the same time point out that the smaller we assess the potential difference between paternal and maternal chromosomes in relation to the normal somatic charge, the greater will be the residual charge.

The next division is of the ordinary somatic type, but with this difference that there are now only half the somatic number of chromosomes, and therefore only half the number of chromatin granules in each cell. It is quite evident, therefore, that in this somatic division the respective charges cannot be neutralised, for we have the whole mechanism of mitosis but only half the proper number of chromatin granules. Following out the case above, the reduced number of chromosomes is two, and each will give two daughter chromosomes, carrying one-quarter positive and negative charges respectively, which are the normal somatic charges for the individual in question. At the end of this mitosis there will be again a balance of charge left over, for in one daughter cell there is one unit positive charge and two one-quarter unit negative charges, leaving a balance of a half-unit positive charge; similarly, in the other daughter cell there is a balance of a half-unit negative charge.

A second case, which frequently occurs, is the formation of the tetrads, and in this case, as in the previous, the result is the same—a balance of charge must be left in the daughter cells. The formation of the tetrads is due, no doubt, to the somatic division of the chromosomes taking place before the first division has proceeded very far. Assuming, as before, the existence of one-eighth unit-charge upon paternal and maternal chromosomes respectively, the first stage will be the formation

of the bi-polar gemini. These gemini will again arrange themselves across the equatorial plate of the mitotic figure and then will begin to undergo the somatic resolution in readiness for the second division. Owing to the bi-polarity being most pronounced at each end of the twin chromosomes this resolution, under normal circumstances, should begin in the centre of each twin, which thus forms a ring, the procedure being of the true hetero-typical kind. This resolution, however, will be accompanied by the establishment of a positive and negative charge respectively on each side of the ring; and, in our case of four somatic chromosomes, this charge will in each case amount to one-quarter unit-charge. This ring formation is followed by the completion of the somatic splitting and the resolution of the gemini back again along the lines of the preliminary fusion. Each ring thereby breaks into four portions, which form the tetrads, and the two cell divisions rapidly take place in succession. The distribution and balance of charges follow exactly the same lines as before, with the establishment of three-quarter unit positive and negative charges in the two first daughter cells and the further establishment of a residual half positive and negative charges alternately in the grand-daughter cells. The only proviso that we must make as regards maturation by tetrads is that the two divisions follow one another in rapid succession with no intervening resting stage. This, however, seems quite a reasonable position to take up, viz., that when the somatic division of chromosomes takes place with the formation of tetrads during the first maturation division, the second maturation division must follow the first at once. If the somatic resolution does not take place, or only takes place incompletely, then the charges will be the same whether there is a resting period betwen the two or not.

Before discussing the result of the establishment of the residual electric charges in maturation, it may be pointed out that the hetero-typical resolution of the meiotic gemini with the formation of rings will only take place when all the chromatin granules in each twin are perfectly uniform. We might readily imagine that the chromatin granules in one paternal chromosome, for example, are weaker in character than those in the maternal chromosome with which it fuses to form a twin. In this special case the ring would not necessarily be formed when the somatic split took place, but rather, a body of the form:

 or 

Moore and Arnold have described various forms of these gemini in the meiotic phases of many germ cells, and it would seem that if their existence be confirmed they can be explained by certain distributions of activity in the chromatin granules in the twins. They are, therefore, only of secondary importance as far as regards the phenomena under consideration.

It may be argued that the formation of residual charges might be prevented by the cytoplasm providing in each division centrosomes of just sufficient charge to meet the needs of such division. This, however, seems impossible of belief, for it would mean a variation in the power of the cytoplasm between very large limits in a very short space of time. Against this view, we would point out that the cytoplasmic activity of the germ cells at the time of maturation is exceedingly great, and, therefore, it seems in the highest degree unlikely that the resolving power should become half the normal value or even less, and, further, that it should vary. Although it may be said that we have arbitrarily assumed a difference of potential between paternal and maternal chromosomes and fixed it at one-eighth of a unit-charge, it must be remembered that whatever be the view taken of it, the establishment of residual charges is necessary, for only one somatic division of the chromatin granules occurs and twice the somatic number of centrosomes are brought into play. On these grounds alone, without making any assumption whatsoever as regards the existence of the potential difference between paternal and maternal chromatin being necessary to cause the preliminary fusion in pairs to form the meiotic gemini, it appears absolutely impossible for the residual charges not to be established in the maturation divisions. At the same time, as we have already shown, it is necessary to assume some type of difference of potential between paternal and maternal chromatin, and the existence of one of electrical type, as we shall presently show, is quite easy of acceptance, although its value cannot be directly estimated in the present state of our knowledge. If we, therefore, put this at half the somatic charge on the daughter chromosomes, that is to say, in our example of four somatic chromosomes, one-eighth of a unit-charge of positive and negative electricity on paternal and maternal chromosomes respectively, and follow out the mounting up of the residual charges in the maturation divisions, we arrive at the following values:—

In the first, or meiotic, division, the residual charges will be three-quarters of a unit positive and negative charge respectively, for the centrosomes each carry one unit-charge, and there are two chromosomes

with one-eighth charge on each. In one daughter cell we have, therefore:—

$$+1+\left(-\tfrac{1}{8}-\tfrac{1}{8}\right)=+\tfrac{3}{4}$$

and in the other we have:—

$$-1+\left(\tfrac{1}{8}+\tfrac{1}{8}\right)=-\tfrac{3}{4}$$

The second, or post-meiotic, division proceeds naturally quite independently of these charges, and as we have before shown, owing to there being only half the number of somatic granules, a further charge is established in each grand-daughter cell of half-unit positive or half-unit negative charge. To determine the total residual charges in the grand-daughter cells it is necessary to add the pre-existing residual charges in the daughter cells to those formed in the grand-daughter cells. To one pair of the latter, which carry a half-positive and negative charge respectively, we must add the three-quarter positive charge. To the other pair we must add the three-quarter negative charge, so that the total residual charges in each set of four grand-daughter cells are one-and-a-quarter positive, one-quarter positive, one-quarter negative, and one-and-a-quarter negative respectively. This is perhaps shown more clearly in fig. 3.

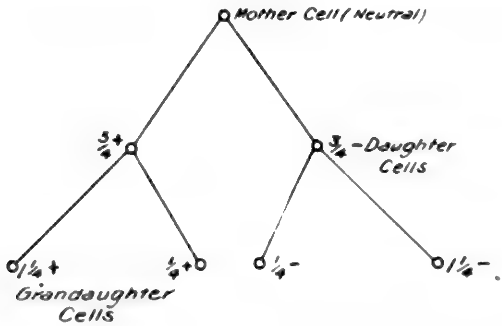


FIG. 3.

It may be pointed out here that the amount of the residual charges is entirely independent of the somatic number of chromosomes. We have, purely for purposes of argument, considered the special case of four somatic chromosomes. Exactly the same results and the same quantities of residual charges are obtained, as may readily be seen, whatever be the number of somatic chromosomes.

Now, in the case of the maturation of the ovum, only one of the

grand-daughter cells is utilised, the other three being rejected as the polar bodies; but in spermatogenesis each of the four grand-daughter cells produces a functional spermatozoon, so that we arrive at the interesting result that one of the spermatozoa is of the same type as the ovum and one of the exactly opposite type; while the other two are of an intermediary type.

Herein, in all probability, lies the secret of sex production and the explanation of Mendelism, for it would seem perfectly natural that if the ovum be fertilised with the spermatozoon of the same type, a female embryo will be produced; if with spermatozoa of opposite type, male; and if with either of the two intermediary spermatozoa, a heterozygote will be formed. We use the word heterozygote in the Mendelian sense, and would mean either a male embryo, which is not entirely male, but one with male characteristics predominating over the female; or female embryo in which female predominates over male. On the above lines, it is evident that the essential of sex is primarily established in the meiotic division, for it is here that the first residual charges are established. The post-meiotic, or somatic division, merely alters the proportion in which the male and female characters predominate.

These results afford a simple explanation of Wilson's[1] experiments upon the insect *Protenor belfragi*, without any assumption of selective fertilisation. The male Protenor possesses five chromosomes in its somatic cells, while the female has six, and as there occurs an irregular distribution of chromosomes in the spermatogenesis of the male, it is possible to follow the results of fertilisation with greater accuracy than in an ordinary case. Wilson finds that in the spermatogenesis the odd, or hetero-tropic, chromosome does not fuse with any of the other chromosomes, but passes bodily over into one of the daughter cells of the meiotic division. In the post-meiotic division this hetero-tropic chromosome divides as usual, so that of the four grand-daughter cells two give spermatozoa with three chromosomes and two give spermatozoa with two chromosomes. In the case of the oogenesis of the female, there is no irregularity, and the ovum possesses naturally three chromosomes. When the ovum is fertilised by a spermatozoon containing the hetero-tropic chromosomes a female is always produced, while if the fertilisation take place by one of the spermatozoa containing two chromosomes a male is always produced. Now, on the theory which we put forward, the male embryo receives two chromosomes from the spermatozoa and three from

1. *Science*, N.S., XXIII, p. 500 (1905).

the ovum, and when, therefore, the spermatogenesis of this individual takes place, the two paternal chromosomes fuse with two of the maternal chromosomes, giving meiotic gemini, leaving the third maternal chromosome over as the hetero-tropic one. When the splitting of the meiotic gemini takes place the two maternal portions go to one daughter cell and the two paternal portions to the other. The hetero-tropic chromosome being of maternal origin will go along with the two maternal halves of the gemini into the same daughter cell. It is this cell which, by virtue of its residual charge, has established in the female predominance, so that when one of its daughter cells fertilises the ovum the female will always be produced. Similarly, the other daughter cell of the meiotic division has male characteristics formed on it by its residual charge, so that its daughter cells always give male insects in fertilisation. Wilson attempted to explain these facts by means of attributing a female determinancy to the hetero-tropic chromosome, but owing to the thereby necessary existence of the male determinant, it became necessary to assume a selective fertilisation; this assumption, which, from first principles, seems improbable, is therefore rendered entirely unnecessary.

It is only right to point out that McLung[1] was the first to suggest from the investigations upon the accessory chromosome that the sex determinancy lies in the spermatozoa. To quote McLung's own words:—'I must here also point out a fact that does not seem to have the recognition it deserves, viz., that if there is a cross division of the chromosomes in the maturation mitosis, there must be two kinds of spermatozoa regardless of the presence of the accessory chromosome. It is thus possible that even in the absence of any specialised element a preponderant maleness would attach to one-half of the spermatozoa, due to the qualitative division of the tetrads.'

The differentiation between the cells produced in the maturation division appears to give also the key to the problems of parthenogenesis. In the first type of parthenogenesis, as in Aphis, when only one polar body is formed the female only is produced. As we have already shown, the meiotic division establishes the sex of the cell, so that in Aphis the cell with the male character, established in it by virtue of its residual charge, is rejected as the polar body; the nucleus remaining contains the female character established in it, so that when this egg develops parthenogenetically a female insect must be produced.

1. C. E. McLung, 'The Accessory Chromosome: Sex Determinant?' *Biological Bulletin*, III, p. 43, 1902.

The second type of parthenogenesis, as occurs for example, in the honey-bee, where fertilisation takes place by means of the second polar body, the egg always produces a male, while the eggs fertilised by these males always produce workers, which are, of course, female, but do not develop into queens unless specially fed during their early stages of development. On the theory of the differentiation between the four grand-daughter cells of the maturation division, the fertilisation of the ovum by the second polar body becomes at once comprehensible, for this polar body bears exactly the same relation, as regards its residual charge to the ovum, as does one of the spermatozoa; indeed, the polar body must be of male character towards the ovum, so that inasmuch as the fertilisation takes place within the one individual a male embryo is produced. This means that the female characteristic established by the first or meiotic division does not alter the actual relations between the polar body and the ovum, a view which is supported by the fact that the cleavage centrosomes arise from the cytoplasm of the ovum. Furthermore, inasmuch as the fertilisation is carried out under the aegis, so to speak, of the female dominance, the male produced will undoubtedly be heterozygote, moreover, heterozygote with an unusually large presence of female character. The spermatozoa eventually produced by a male of this type will clearly be of different character from those of the hypothetically true homozygote male. So far is the female character present that he cannot beget any but female offspring—a fact not at all incomprehensible if it be remembered that the queen bee is an unusually highly developed female. It is not surprising, therefore, that the females normally produced from the fertilised egg are undeveloped, and it at once raises the question whether they are all exactly the same. As a result of this explanation, it would be expected that the worker bees possess among themselves differences in their potential powers of development into queens. Indeed, it might be expected that the special feeding might fail in developing queens from a small percentage of the worker larvae. The important point, however, for the present purpose is the fact that the present theory gives a reasonable explanation of the fertilisation by the second polar body.

The point was raised above as regards the sex characters of the spermatozoa of the heterozygote male, and it was stated that the relations between the charges of the spermatids in each quartette would be altered. This deduction follows reasonably from the general theory, and if, for example, a male was heterozygote to the extent of three parts male and

two parts female, it is probable that the intermediary spermatozoa would be altered. It is quite conceivable, for example, that the spermatozoon, which in a normal case would give a heterozygote male, might be so far altered by being itself produced from a heterozygote as to become potentially female in character, so that, on fertilisation, a heterozygote female would be produced. On the average, a homozygote male and female would produce equal numbers of male and female offspring, but a heterozygote male would beget a greater proportion of female offspring.

Amongst the human race this may possibly account for the large predominance of male or female children which frequently occurs in certain families. Again, it is not impossible that the condition of health that the parent may be in at the time of maturation may influence the value of the residual charges in the grand-daughter cells. If the vitality were low, it might readily follow that the residual charges be reduced so far that the relative dominance be affected. This would account for the results of the experiments upon the feeding of one parent and starving the other in the case of certain amphibia, etc., when it was found that the sex of the stronger parent predominated in the offspring.

We have taken for granted in the case of the oogenesis of the female that the ovum is the one grand-daughter cell of the mother cell, which is the true homozygote female. Our reason for this is simply that it seems the most likely condition to occur, though there seems no valid reason why the other conditions should not obtain. Even if one of the other conditions were to take place, it would not materially alter any of the conclusions drawn above.

It must be remembered that the post-meiotic division tends to increase the residual charges in the resulting cells, and therefore it would follow that the fact of there being no further divisions after the post-meiotic one is due to the residual charges having already reached the limiting value. There naturally must be a limit to the power of the cytoplasm and chromatin in building up these residual charges, and, no doubt, this limit is reached in the post-meiotic division.

While it is by no means necessary, therefore, that the charges are of the actual value we have arrived at, yet the establishment of some type of residual charges seems an essential consequence of an electrical theory of mitosis, and moreover these charges can, in a satisfactory manner, give an explanation of the phenomenon of sex production.

Although it is probable in the main that the maternal chromosomes pass during the meiotic division into the female daughter cell, yet there

is no doubt that during the existence of the meiotic gemini, some re-distribution of the chromatin granules takes place. In order to account for the Mendelian distribution of hereditary characteristics, it is essential that the chromosomes formed by the splitting of the meiotic gemini be not necessarily the same individuals as originally fused together; there must be an interchange of the granules during the time of fusion, according to Dr. Vries's theory. This, however, does not concern the present purpose very much, for whether this takes place or not, no fundamental change in the relative charges will occur. It must be remembered, however, that any re-arrangement of the granules in the meiotic gemini will tend to decrease the potential difference between the maternal and paternal portions, and, therefore, the potential difference between the two chromosomes obtained when the gemini are split. From a consideration of the residual charges obtained in the first maturation or meiotic division, it is evident that the smaller the potential difference between the split chromosomes of the gemini, the greater the residual charge or sex determinance in the resulting daughter cell. This affords a reasonable explanation of the De Vries re-arrangement of the chromatin granules, viz., the decrease in the potential difference between the two halves of the gemini. For there is no doubt that the re-arrangement will take place, since such re-arrangement tends to decrease the potential difference. We may say, therefore, that the De Vries' re-arrangement of the granules is not a matter of chance; it is a direct consequence of the potential gradient existing within the bi-polar twin, and the number of individual granules which interchange will be determined by the steepness of this potential gradient. In other words, the greater the potential difference between the paternal and maternal chromatin, the greater the number of interchanges among the granules, and hence, also, the more pronounced become the sex characteristics of the grand-daughter cells.

Now there remain certain other facts in connection with fertilisation which are brought into co-ordination by this theory. In the first place we have the very profound changes which are induced in the cytoplasm of the ovum at the time of fertilisation. Space will not permit of our dealing with these in detail, but they can, in general, be explained by chemical and physical reactions taking place when the charge on the spermatozoon enters the ovum. It must be remembered that the density of the electric charge on the spermatozoon is very much higher than that on the ovum, owing to its being so excessively minute compared with the ovum, and the fact of its sudden introduction will be to cause a wave of energy to travel

through the cytoplasm. Again, the difference in polarity between the ovum and the spermatozoa nuclei will account for the different staining reactions which have at times been observed between the two, for a charge of positive electricity on a mass of protein material will increase its basicity, while its acidity will be increased by a negative charge. After fertilisation, as the charges become equally distributed, the staining reactions of the two nuclei will approach more and more nearly together.

Perhaps one of the most important results of this theory is that it leads to the view that the ovum and spermatozoon are both cells with residual charges established in them, and which cannot reproduce themselves of their own power, owing to these charges. The ovum, moreover, does not possess any material wherewith to form centrosomes and so start mitosis, while the spermatozoon, although it possesses a centrosome in the middle piece, has no cytoplasm to start the mitotic machine: when the two are brought together, the machinery is completed and mitosis can start, provided, of course, that the temperature is sufficient. We are, therefore brought to the conclusion that the chromosomes are individual entities, preserving their individuality all through each cycle, and that the centrosomes are of paternal origin. What the latter consist of, it is idle to speculate, beyond saying that they are, in all probability, discrete granules of protein, considerably simpler in character than the cytoplasm, which can be resolved by the cytoplasm into two parts of basic and acid type with a positive and negative charge respectively.

It follows that the fertilisation of the enucleated ovum will give rise to an embryo (with, of course, only half the normal number of chromosomes), this being the natural result of the theory. There is no essential need for both nuclei to be present, since the machinery is complete for mitosis when the spermatozoon enters the cytoplasm of the ovum. The converse of this, or the artificial fertilisation of an ovum, also follows, and is equally easy of explanation. The most striking case is that of Loeb, who succeeded in causing sea-urchin eggs to develop parthenogenetically by placing them in a solution of magnesium chloride. If a colloid mass be placed in a solution of an ionised salt, the osmotic pressure of the ions will be exerted upon the surface of the colloid. In time, as the water penetrates a little into the colloid, the ions, owing to their diffusion velocities, will tend to penetrate the surface. The velocities of the two ions, however, are different, so that one will always be very slightly ahead of the other, with the result that the surface of the colloid will tend to become electrified. Thus, when the eggs are put into

magnesium chloride solution the outer layers become electrified, and even though this effect be produced at only one small portion of the surface, yet, with the previously existing charge of the egg, it is enough to cause parthenogenetic development. The first result will be much the same as when the spermatozoon normally enters, viz., the same modification of the cytoplasm, which is doubtless due to the re-distribution of the charges. The production of the centrosomes would appear to arise from some portion of the cytoplasm which is coagulated, and, possibly, hydrolysed to a certain extent by the electrical and chemical stimulus given by the ions. The parthenogenetic development of the eggs of the silk worm noticed by Tichomiroff[1] on gently rubbing them with a brush is capable of the same explanation, namely, the electrification of the surface layers of the cytoplasm.

The question may be raised here as regards the continuity of the centrosomes through the life of any individual, and it may be pointed out that from the point of view of the electrical theory, there is no *prima facie* evidence why they should possess any continuity. The disappearance of the old centrosomes and the appearance *de novo* of another set is perfectly possible. They may arise from the old spermatid centrosome in the middle piece of the spermatozoon, or from one of the vesicles of the archoplasm. The same centrosome may go on dividing itself up continuously all through life, or at any period a new set may arise as far as the theory is concerned. It is not probable that any change in the centrosomes will arise after several generations of cell division have taken place, for the number of available particles introduced by the spermatozoon get fewer owing to their absorption or distribution. In the first few generations a new set of centrosomes might easily arise and, indeed, have been seen to do so.[2]

There is another point which may be mentioned, and which gives some support to this theory, namely, that the second polar body is sometimes not formed in the ovum until after the entrance of the spermatozoon, and, indeed, in Chaetopterus[3] the first polar mitosis stops at the anaphase until the sperm has entered, when the mitosis is resumed and both polar bodies are formed. The explanation of these facts on the electrical theory is that the cytoplasmic power is not sufficient to carry out the maturation divisions and establish the residuai charges. When the tension is relieved

1. Loeb, *loc. cit.*, p. 165.
2. *Morgan Rept. of the Amer. Morph. Soc. Science*, III (1896).
3. *Med. Journ. Micros.*, X, p. 1 (1895).

by the entrance of the spermatozoon then the division proceeds normally. This is a strong support of the view that polarity or stress is established by the maturation division and that it is relieved on the entrance of the spermatozoon [1] Mead has noticed that the eggs of Chaetopterus throw out their polar bodies if a little iodide of potassium be added to the sea-water. This is quite analogous to the artificial parthenogenesis discussed above, for the influence of the potassium ions relieves the tension set up by the maturation divisions in the same way as does the spermatozoon, and the two divisions can complete themselves in the normal way.

Strong support for the electrical theory is also to be found in the fact that both males and females are rendered completely sterile by X-rays. The action of X-rays in breaking down the di-electric is well known, and in these cases of sterilisation by X-rays the cell material becomes a conductor and the residual charges are dissipated, with the result that both spermatozoa and ova are defunctionated. These facts offer the most striking support to the theory.

There remains now only to be considered the assumption made in the section dealing with maturation that a definite potential difference exists in the germ cells between paternal and maternal chromatin. As a matter of fact, there is no doubt that there is a considerable difference between the paternal and maternal chromatin, which may very possibly be established by the residual charges in the ovum and spermatozoon, for whatever may be the actual value of the residual charges produced in maturation, the greater density of charge on the spermatozoon relatively to that on the ovum must be borne in mind—a result which is due to the relatively minute size of the spermatozoon. It is possible that this difference may be the origin of the potential difference between the paternal and maternal chromatin in the germ cells, for though there is an equal distribution of the electricity tending towards neutrality at fertilisation, yet it is quite reasonable to suppose that perfect neutrality is not at once established, or, in other words, that paternal and maternal chromatin do not at once become identical. That it becomes so eventually in the somatic cells must indeed be the case, while it is not a great assumption to make that the potential difference is preserved in those cells destined to become germ cells. If this were the case, it is evident that there should be some differentiation between the somatic cell ancestors and the germ cell ancestors from the very beginning of the cleavage

1. *Lectures at Wood's Hole, Boston*, 1898.

nucleus. Indeed, Boveri[1] has found such a differentiation in Ascaris at the two-cell stage, for in the cell destined to give rise to the somatic cells there is evidenced a definite rejection at each division of some of the chromatin, while in those cells destined to form the germ cells this does not occur. It would appear from this that the casting out of some of the chromatin in the early somatic cells is the means adopted to establish the necessary complete neutrality between paternal and maternal chromosomes, while in the germ cells this neutrality is not established and the potential difference remains, but of too small an amount to make itself felt. As the individual grows, and the sex becomes established, the originally existing difference of potential would tend to become emphasised until it becomes sufficiently large to interfere with normal mitosis; the cells then enter their long resting stage only to emerge with the formation of the meiotic gemini.

An interesting point arises in connection with the fact that in the animal kingdom only one somatic division occurs after the reducing division. It is evident from the theory put forward of the electric residual charges that the establishment of these charges means a certain condition of stress, and it therefore follows that the work done by the organism in carrying out a maturation division must be greater than that in a somatic division. If we consider for a moment the driving force which works the somatic machine, we can clearly equate this to the integration or sum of all the small amounts of work done in each cell division. The energy used and the work done are undoubtedly commensurable and capable of expression in terms of some simple unit. There is no doubt, therefore, that the force available for any one cell division is limited, and it would appear that the limit is reached at the end of the post-meiotic division. The available force is not sufficient to carry out any further divisions resulting as they would in still further enhanced values of the residual charges.

It is no essential part of the theory here put forward that there should be one, and only one, division of the somatic type after the meiotic or reducing division. As we pointed out above, when this state of things occurs it would mean that the available energy is not sufficient to carry out another division—the limit has been reached. If, however, the stresses set up by the two maturation divisions were smaller in proportion to the driving force then the limit would not necessarily be reached at

1. Boveri, 'Befruchtung,' *Merkel und Bonnet's Ergebnisse*, 1891.

the end of the post-meiotic division, and thus further divisions would ensue until the limit is reached. The number of divisions following the reducing division should be proportional to the ratio of the driving power to the stresses set up in the reducing division.

In advancing our theory of the electrical forces of mitosis we have discussed the phenomena as present themselves in the higher animals wherein the sex characteristics are as marked and as differentiated as possible. Clearly we should find a completely graduated scale down from the animals with complete sex differentiation to the lowest organism where no sex differentiation exists at all. Following on the lines laid down in this paper we would say that the complete establishment of sex differentiation means the establishment of certain definite electric charges in the germ cells during maturation, and that the magnitude of these charges is such that only one post-meiotic division can occur. If the sex characteristics are not so strongly developed then the charges established in maturation will not be so great in relation to the driving force, with the result that more divisions will occur before the limit is reached.

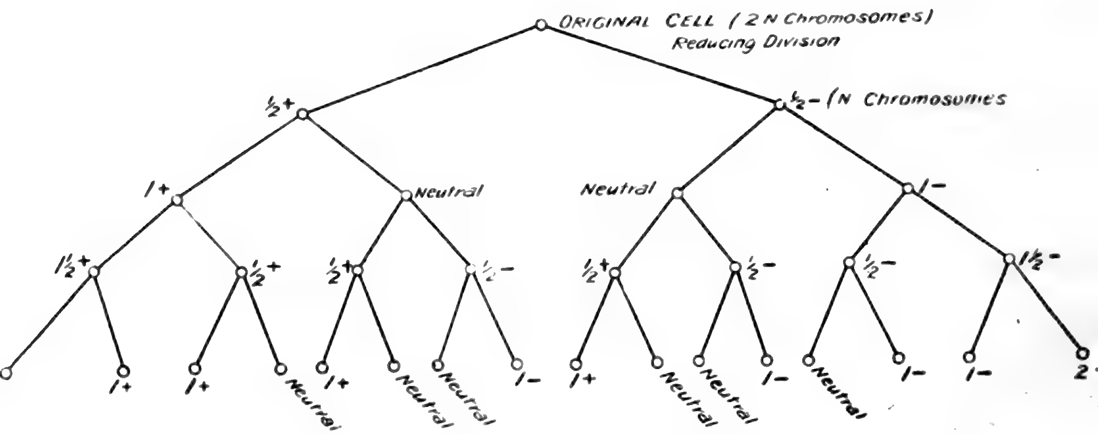
We may form some idea of the evolution of sexual differences from the standpoint of the electrical theory without any assumption beyond those already made. The essential point is, of course, the first inception of the reducing division, why it first occurred and why the fusion of two cells carrying the reduced number of chromosomes takes place. If we consider a single living cell such as that of a unicellular organism with no very well defined characteristics (using the word in the hereditary sense), it is natural to imagine that this organism can reproduce itself by ordinary mitosis without any of the sexual phases. When, however, in the process of evolution more characteristics are acquired we at once expect a break in the continuity of reproduction. *When an organism possesses characteristics of opposite polarity it is an absolute necessity that a reducing division shall sooner or later take place.*

As long as the acquired characteristics are of the same type an organism can reproduce itself mitotically without difficulty, but when the chromatin granules or chromosomes carrying the characteristics show opposite polarity, a reducing division must occur, because sooner or later the difference of polarity between the chromosomes will reach a value sufficiently large to cause the fusion of them together to form the bi-polar twin. This will be followed by the so-called reducing division. Now

this division sets up the residual charges, however small they may be, and these will go on slowly mounting up in the following divisions until they are of sufficient size to cause the fusion or union of two cells (of opposite charge) with the restoration of the original number of chromosomes.

During the process of evolution, as the acquired characteristics become more numerous, they will by virtue of their different polarity be segregated more and more on the one side and the other until finally we have two different individuals—the male and female.

It is interesting to follow out the cell divisions as might be expected to occur in cases where the sex differentiation is small. We may consider an organism with $2n$ chromosomes which carry characteristics of not very marked opposite polarity. The cells will go on dividing normally until the opposite polarity is sufficiently great to cause the fusion of the chromosomes with formation of the bi-polar gemini followed by the reducing division. The new cells will now have n chromosomes, and these will go on dividing with a balance of charge left at each division. If now the balance of charge left in each daughter cell at each division for purposes of argument be put at $\frac{1}{2}+$ and $\frac{1}{2}-$ respectively, then the following values will be obtained:—



As can be seen from the diagram, there will be, after the third post-meiotic division, sixteen cells, each carrying n chromosomes, and of these six will be neutral, four will have a unit positive charge, four a unit negative charge, one 2 units positive and one 2 units negative electricity

respectively.[1] If the distribution of charges be followed out for the ensuing divisions, it will be found that two of the cells formed always carry equal maxima of positive and negative electricity respectively, and there will continually be produced a constant ratio of neutral cells and cells with intermediate values of charge. Sooner or later the magnitude of the charges produced will cause the maximum charged cells to fuse together with the formation of new cells carrying $2n$ chromosomes, when, of course, the cycle begins again. The organism, therefore, will present the appearance of having two types of cell, one carrying $2n$ and the other n chromosomes. Some of the latter will from time to time fuse together to form new cells with $2n$ chormosomes, and so the cycle is complete. This state of affairs will be the natural result of the sex differentiation being very incomplete, or, in other words, when the hereditary characteristics have not developed the maximum possible difference in polarity. When this occurs we find the condition that, owing to the magnitude of the charges involved, only one post-meiotic division takes place, the cell being at once sufficiently charged for fusion or fertilisation by one of opposite type. *The whole difference between the various types of reproduction may therefore be summed up in the conception of sex differentiation, or of opposite polarity in the hereditary characteristics.*

It is also of some importance to note that as sex differentiation is increased the relative size of egg to sperm is increased. When the sex differentiation is small, or as we might say merely embryonic, there will be no difference in size or visible character of male and female cells. When the male and female chromatin begin to differ materially then we find a difference in the development of the two, the male becoming smaller than the female, until finally we arrive at the spermatozoon and ovum, where the ratio of size is very marked indeed.

In the development of the above argument we have attributed the variation in the number of post-meiotic divisions, i.e., divisions between the reduction and the ensuing fusion, to the ratio between the driving force and residual charges established; the greater this is, the greater the number of post-meiotic divisions. We have introduced the conception of driving force merely to illustrate our point, and would now deal with this conception in more detail and consider the relation of the chromatin to the surrounding nucleic acid. There is no doubt that during the resting period between two successive divisions the chromatin must be growing

1. Exactly the same relative values are obtained, whatever be the size of the residual charges.

by means of chemical reactions between itself and the surrounding nucleic acid. Indeed, we may go so far as to say that unless this growth occurs any somatic division is not possible, for such division would result in a decrease in the active mass of chromatin in the daughter cells, a consequence impossible to accept. Now from a physico-chemical point of view it is evident that any reaction between chromatin and nucleic acid must be based upon some essential points of similarity in structure between the two, and it would seem a natural deduction to make from this standpoint that any influence tending to decrease the similarity between the two would retard the growth of the chromatin at the expense of the nucleic acid. Hence it is quite a natural sequence that a decrease in the similarity would tend to act as a deterrent to mitosis. The establishment of the residual charges in the maturation divisions, of course, means a considerable modification in the chromatin of the daughter cells, i.e., a considerable decrease in similarity between the chromatin and nucleic acid, so that we finally arrive at the conclusion that the establishment of the residual charges is a direct deterrent to mitosis. Hence the failure in the animal kingdom of the daughter cells of the post-meiotic division to undergo further divisions may be explained at once by the fact that the residual charges prevent the growth of the resulting chromatin, so that no reason or scope for division exists.

We arrive, therefore, in this way at a very definite foundation for the assumption made previously that the number of post-meiotic divisions depends upon the ratio of driving force to amount of residual charges established, for we find that the driving force is essentially determined by the growth of the chromatin during the so-called resting period. The influence of the amount of differentiation between male and female characteristics upon the number of post-meiotic divisions follows very clearly from this view, for, as several times pointed out, the amount of residual charge in the meiotic division depends directly upon this differentiation, or, as we would now state the law: *The greater the number of different hereditary characteristics of opposite type the greater is the dissimilarity between the chromatin and nucleic acid in the grand-daughter cells of the reducing divisions, and hence the fewer the number of post-meiotic divisions.*

It may be stated here, parenthetically, that whatever view be taken of the method by which the chromatin grows or the period at which the growth occurs, the same conclusion is arrived at: that if the growth be stopped then mitosis cannot occur.

It is impossible to enter fully into the various types of reproduction which are known to occur, especially in the vegetable kingdom, but there seems no doubt that they all are capable of explanation on the theory here put forward. We have dealt with the case of the unicellular organism with no sex characteristics, those cases in the vegetable kingdom where incomplete sex differentiation exists, and the zoological cases where the sex differentiation is complete with the two sexes as distinct as possible. All other cases seem only to be intermediate between the first and last.

A very important corollary must be added to what has been said above upon the reducing divisions. At the completion of this division there is established a definite amount of residual charge in the daughter cells. In other words, a certain amount of energy is stored in the cells, and this must result in the vitality of the cell being increased. This fact is very important, for it would seem that the reducing division forms a means by which the vitality or activity of the cells is renewed, for there is no doubt that a considerable amount of energy is absorbed at the time.

Hitherto we have made the tacit assumption that the phenomena described are those occurring in the mitosis of normal healthy cells. The question now arises as to what would happen if the electric charges in the somatic cells were disturbed by some means, and the equilibrium between them upset. It is very evident that important changes in the phenomena might take place, and it has occurred to us that the various types of malignant growth might very readily be explicable by their being due to the derangement of the electrical forces present during mitosis. On studying the experimental results obtained in this field we were very forcibly struck by the support given to our idea, and we feel more than justified in offering this as a reasonable explanation of malignant growths, viz., that they are due to abnormal cell reproduction arising from a disturbance of the electrical equilibrium of mitosis.

One of the simplest methods of causing a disturbance of the electrical equilibrium in the cell is by the external application of an electrical stimulus. If, for example, a somatic cell was given an added charge, i.e., if the cell wall by some means were electrified, a natural sequence would be an artificially produced multi-polar mitosis as already described in the fertilisation experiments of Boveri and Loeb above. Furthermore, the daughter cells produced as a result of such mitosis will naturally have a balance of positive or negative electricity left in them, owing to the result of the asymmetric distribution of the chromosomes. Since there is no means of dissipating these residual charges, the effect of a

single initial external stimulus will be handed on from generation to generation of daughter cells. There is no doubt that the periodic activity of the cytoplasm resulting in its mass division would not be interfered with by the small external stimuli referred to. The mechanism of the cell mitosis would be the same in the main, but owing to the excess of electrical energy multi-polar and asymmetric mitoses must result.

Now, multi-polar and asymmetric mitoses are frequently observed in malignant growths, and, indeed, Galeotti has artificially produced asymmetric mitoses in Salamander cells by treating them with certain chemical substances.[1] These substances undoubtedly acted as a stimulant to the cell either by electrification of the cell walls by virtue of the different velocities of the ions, as already shown in the case of Loeb's experiments, or by more purely chemical means. That the action of an external stimulus is capable of producing these two pathological mitoses in cancer, by which term we understand all malignant neoplasms, is thus evident. Waller[2] and others have shown that electrostatic differences of potential are a normal result of any external stimulus being applied to healthy tissue, and whether we accept Loeb's[3] explanation or not that the effect is due to the migration of hydrogen ions, still the fact of sufficient potential difference being set up to deflect a galvanometer is completely established. It is possible, therefore, that an external stimulus, as for an example a bruise or blow, could induce sufficient electricity to derange all the neighbouring cells, that is to say, a sufficient electric stimulus could be established to start pathological mitoses. It would thus appear that, provided the necessary conditions were existent, a blow or bruise could give rise to a malignant growth. It stands to reason that the more healthy is a cell and the stronger its vitality, the greater will be its resisting power against the effect of an external stimulus. Conversely, the lower the vitality of the cell the more liable it becomes to derangement. There is no doubt that, speaking generally, the vitality of cells must decrease with their age, so we would expect the tendency to malignant growths to increase with age: in other words, we have herein a direct explanation of the age incidence of cancer. The actual change which takes place, and which we have spoken of as a decrease in vitality, would be due partly to a decrease in the active growth of the cytoplasm and partly to a decrease in the active growth of the vital units or chromatin

1. Beib. 3, *Path. Anat.*, XIV, 2 (1893).
2. Waller, *Signs of Life*, p. 143.
3. Loeb, *loc. cit.*, p. 68.

granules. Whereas in a healthy cell the mitotic phenomena are due to forces which are periodically brought into play, so a decrease in these forces will tend to weaken the vitality of the cell. We have shown, also, that the possibility of mitosis is dependent upon the growth of the chromatin, and as this certainly will decrease with the age and differentiation of the cell, so the vitality of the cell will decrease with age. We therefore have two causes for the decrease in the vitality of a cell.

It is not possible in the present state of our knowledge to advance any definite chemical theory for the change which is developed with age, but it is fairly certain from cytological investigations that a stage is reached when the cytoplasmic power is not sufficient to resolve the centrosomes and chromosomes with the formation of the mitotic figures, and then amitotic division may and does frequently occur. It may, however, be concluded that the occurrence of amitosis is due to the fact that the active growth of the chromatin has fallen to a value below the limiting value for mitotic resolution of the chromatin granules while the cytoplasmic activity is sufficiently great to cause the mass division to take place. We have already directly connected the decrease in vitality of the cell with the occurrence of cancer, so the ocurrence of the amitotic divisions of cancer cells is easy to understand. As already pointed out, the age incidence of malignant growths is the natural sequence of the fact that the somatic cells must reach a certain critical minimum of vitality before they can be disturbed by any external stimulus. We may for the present purpose give the name of the nth generation to that generation at which the cells reach the critical minimum, i.e., when their vitality has fallen low enough to be susceptible of disturbance by the external stimulus. It is of some importance at this point to notice that when any tissue is subject to continually repeated abrasion or irritation, the resulting continuous renewal of tissue will cause the nth generation to be arrived at somewhat earlier than would otherwise be the case. From Waller's experiments it is a natural sequence that any irritation or stress should tend to produce an electrical difference of potential. This would seem at once to account for the occurrence of malignant growths in those parts of the body which are subject to such stresses.

Hitherto we have been considering the derangement of a cell by external stimulus, but it is also possible that derangement may occur internally.

If we consider the case of a cell which is highly differentiated, and, moreover, one the vitality of which has sunk very low, it is evident on

first principles that the potential difference, actually set up when the vital units are resolved, has fallen to a very small value. This decrease in activity, sooner or later, as we have before pointed out, results in amitotic division. This occurrence of amitosis, however, demands that all the chromatin granules must be at a low ebb of vitality. The formation of the loose compounds between the nucleic acid and the chromatin granules must, to all intents and purposes, have ceased, or, in other words, the growth of the vital units has become very slow. It is not essentially necessary, however, that every single chromatin granule of the cell decrease in vitality at the same rate, and the question arises as to what would happen if the vitality of the various granules differ considerably within a single cell. If we consider for a moment the effect of inbreeding and hybridisation, it is perfectly evident that by far the most probable condition is the one specified, namely, a considerable variation in vitality among the granules of chromatin within each cell. Clearly this gradation of vitality among the chromatin granules can give rise to a derangement of the electrical equilibrium in mitosis, as can be seen from the following. Whereas in the normal mitosis of a healthy somatic cell the chromosomes give daughter chromosomes of equal and opposite charge, and as in true amitosis the chromosomes are not resolved at all, in the present case a condition may arise when some of the chromosomes are resolved while the remainder are not so resolved, owing to the vitality of the granules of the latter being very much lower than that of the granules of the former. These latter chromosomes would only be polarised, and therefore would not migrate to either centrosome; a fusion of the electrified daughter chromosomes with the polarised chromosomes would naturally ensue, and an asymmetrical mitosis would take place, with a reduction in the number of chromosomes. This would again cause a balance of residual electric charge in each daughter cell, exactly in the same way as occurred in the case of the external stimulus described above.

Further, it must be pointed out that the age incidence would apply to this condition just as much as to an ordinary cell, for the derangement is caused by the vitality of the weakest granules falling below the minimum value and also by a general decrease in the activity, both being the normal results of the age of the cell. To assume for the moment that this condition of chromatin granules is possible, we may now re-state our case as it stands. An electrical theory of mitosis at once renders possible abnormal mitosis by reason of the electrical charges becoming disturbed. The electrical theory of mitosis in itself receives very considerable support

from experiments upon artificial fertilisation, from parthenogenesis, from sterilisation of the ova and spermatozoa by X-rays, etc. The disturbance of the balance of electrical quantities in the cell may arise from the application of an external electrical stimulus arising from a blow or from considerable and repeated irritation or stress. It may also arise internally in the cell from a certain number of the chromatin granules being of such a character that they do not give daughter granules with sufficiently different charges. In both cases the result is the same. The normal equilibrium between the charges is disturbed and asymmetric and multipolar mitoses occur, producing daughter cells with a reduced number of chromosomes and a residual positive and negative electrical charge. The tendency to both these disturbances is increased with the age of the cell.

The main point we have arrived at is that when derangement of the charges occurs, the daughter cells are produced with a balance of positive or negative electricity and that this is accompanied by a reduction in the number of chromosomes. It is, therefore, extremely interesting that Farmer, Moore and Walker[1] claim to have discovered that a true reducing division occurs in malignant growths; that is to say, a division of the same type as the first maturation division of the germ cells. Although these results are still *sub judice*, yet the existence of cells with fewer chromosomes than the normal somatic number seems faily well established. The condition of chromatin granules such as we have postulated would give rise to cells containing any number of chromosomes between the somatic number and half that number, and would not necessitate a true reducing division. For this reason we would give the name of *pseudo-reduction* to this process.

As was pointed out before, in dealing with the reducing division in the vegetable kingdom (that is to say, the cases where the sex differentiation is incomplete) the operation leads to an increase in the vitality of the daughter cells. When, owing to the decrease in the activity of a cell, the pseudo-reducing division occurs, the resulting daughter cells carry a certain amount of residual charges, that is to say, a definite amount of fresh energy has been absorbed by the chromatin. This clearly will enhance the activity or vitality of the cells considered as a whole, and therefore we are met with the condition of a new growth of cells of greatly increased vitality in a tissue of cells of low vitality. It stands to reason that the new cells will multiply with great rapidity and will be out of co-ordination with the soma. Their growth will depend entirely

1. *Proc. Roy. Soc.*, Vol. LXXII (1903).

upon the new lease of vitality which they have received from the reducing division, that is to say, it will be inversely proportional to the age of the cells before the derangement.

The course followed by these new cells should be very much on the same lines as that given in detail upon page 214 for the cells obtained by the true reducing division in the case of an organism with small sex differentiation. The descendants of the pseudo-reducing division of cancer will produce a certain number of neutral cells, a few maximally charged cells both with positive and negative electricity, and the remainder with intermediate charges. It is impossible to assess the relative number of the three types, since the original reducing division was not a true reducing one, but only a pseudo one with an indefinite reduction. The maximum charged cells will soon reach the limit, which cannot be passed owing to the magnitude of the charges involved, while the remainder sub-divide indefinitely. When the maximum charged cells have reached the limit the question at once arises as to their future behaviour. The normal course would be for them to fuse together in pairs of opposite charges, with production of new cells with an increased number of chromosomes. A new generation of highly active cells would thus be formed, and thus the cycle would be complete, just as in the botanical case detailed before. It must also be remembered that under the peculiar circumstances owing to the reaction of the soma there is a supply of leucocytes continuously made. These leucocytes being neutral and active cells would be expected to conjugate with the maximum charged 'reduced' cancer cells, since the electric potential would in this way be reduced. Now Farmer, Moore and Walker[1] have observed such conjugation between leucocytes and cancer cells—an observation which strikingly confirms the theory. No doubt conjugation also occurs with the cells of the surrounding tissue, to which may partly be due the infiltration of the malignant growths.

This seems to us to account fully for the cyclical form of growth of cancer in mice as demonstrated by the Imperial Cancer Research (No. 2, Pt. 2, 1905), but the space at our disposal prevents us from producing further proofs for our contention.

An important point arises here in relation to the age and differentiation of the cell. It would be readily understood that the greater the vitality of any cell, the greater will be its activity in proliferation when the malignant diathesis has once been established. If we suppose, for

1. *Proc. Roy. Soc.*, Vol. LXXII, 1903.

example, that either owing to the abnormal condition of the chromatin granules or the presence of an external electrical stimulus, the electrical equilibrium is disturbed when the cytoplasmic activity is still very considerable, it will be evident that the rate of proliferation will be greater than would be the case had the cytoplasmic activity been less. In other words, the greater the amount of the activity the more rapid the growth of the tumour, the more highly differentiated the cell and the older it is before the taint is established, the less rapid will be the growth of the neoplasm. As the age of the host is increased, therefore, the less potent becomes the taint. If the development and differentiation of the cell has proceeded sufficiently far before the cancerous diathesis is established, the smaller will be the potential differences established by the reduction in the number of chromosomes. The tumour then loses its malignancy to a certain extent, and a cancer of slow growth, such as an atrophic scirrhus, is established. There can be no doubt that the growth of the tumour by reduction in the number of chromosomes produces, as before stated, daughter cells which are out of somatic co-ordination with the host, and the tumour grows parasitically, feeding upon its host. Resulting from this condition of parasitic growth of cells out of somatic co-ordination, the wandering of some of the active cells from the seat of the tumour may occur. When one of these charged cells comes to rest, it possesses potential probability of reproducing itself, resulting in the growth of a secondary tumour of the same type as the primary one. The tendency to the formation of secondary growths must, therefore, depend directly upon the activity of the cells when the taint is first established, because, as before said, the greater the activity the greater the activity of the daughter cells of the pseudo-reducing division.

With reference to this subject we are at once struck with the remarkable facts which have accumulated during recent years with regard to the transplantation of cancerous tissue. As the Imperial Cancer Research have indicated, it is much easier to transmit any malignant growth from a mouse of one locality to a mouse of the same locality than to a mouse of another locality; moreover, there is a general concensus of opinion that cancer can in no way be transmitted from an animal of one species to an animal of another. This is the natural outcome of our view on the cytoplasmic activity.

For the more different the species of the animals in question, the more dissimilar would be their respective cytoplasms; and at a certain limit of difference, conjugation either with leucocytes or normal tissue

cells would be impossible, and the engrafted tumour would not be able to survive the reaction of the connective tissue stroma.

In order that conjugation may take place between a charged nucleus and a neutral nucleus or between two oppositely charged nuclei, it is essential that the limiting surfaces of the two masses of cytoplasm be eliminated at any rate during the process. This elimination of the surface layer can only occur if the two cytoplasmic masses be of specifically the same chemical nature. If, however, as a result of the two individuals having lived in different localities or having been fed with different food, their cytoplasmic material be of different chemical character, conjugation between the nuclei will be impossible owing to the difficulty of eliminating the limiting layers of the cytoplasm of the adjacent cells.

The malignancy of new growths varies generally indirectly with the age of the host. As a corollary to this it may be added that the condition of the chromatin granules may be such that the electrical disturbance occur at a very early age, e.g., in the embryo. The occurrence of sarcoma in utero is doubtless due to this condition, and such cases should be, as they undoubtedly are, exceptionally malignant.

We have hitherto confined ourselves to the mere statement that the existence of chromatin granules of such a type that the daughter particles produced in mitosis have a very small difference of charge will tend to cause electrical disturbances, such as seem to occur in cancer. We may now turn our attention to the investigation of this possible condition, for if this possibility be established it will afford an explanation of the true origin of cancer. In any healthy race with no close inter-marriages, it would appear from first principles that the chromatin granules would be perfectly normal, if healthy female ova are fertilised with spermatozoa of a different type; that is to say, as long as father and mother are sufficiently differentiated and both healthy, the chromosomes of their children and the chromatin granules of their descendants will be normal. If, on the other hand, close inbreeding takes place through several generations, then the chromatin granules will tend to become more and more uniform, so that they give in mitosis daughter particles of smaller and smaller potential difference. The tendency to abnormal mitosis is thereby progressively increased until a stage is reached when the incidence of the malignant growth is markedly intensified.

Up to the present we have not taken into consideration the chemical side of the problem, and although the action of certain chemical

substances has been instanced, as, for example, the production of asymmetric mitoses by antipyrine as observed by Galeotti, the influence of the metabolic products of the cells has not been discussed. We have dealt with the possibility of electrical stimulus and the disorganisation of cells by means of a bruise or blow, and it is a natural sequence of the electrical theory that disorganisation could be produced by a purely chemical stimulus apart from that arising electrically by the different velocities of two ions. Owing to the peculiar configuration of the protein molecule it must be sensitive to both bases and acids, and the influence of these two reactions seems of considerable importance.

As far as is known at the present time the normal cells seem to be strongly influenced by both bases and acids, and the influence of the former seems to be one of stimulation, while acids seem to produce the opposite effect.[1] For example the well-known case of a nerve fibre may be quoted: if two needles be inserted into the fibre and one be connected with the copper of a Daniel cell and the other to the zinc, the nerve cells are stimulated around the latter needle and depressed near the former. It is evident that around the latter there is an excess of alkalinity and an excess of acidity around the former. While several experiments giving similar results might be quoted, the above shows clearly that cells are stimulated by alkali. From a physico-chemical point of view this action of alkali is explicable if the tautomerism or dynamic isomerism of the protein molecule be considered. Recent work has shown that there is a distinct and definite tendency on the part of a - NH - CO - grouping not to exist in either of the two possible desmotropic forms

$$-\mathrm{NH}-\underset{\substack{\| \\ \mathrm{O}}}{\mathrm{C}}- \quad \text{and} \quad -\mathrm{N}=\underset{\substack{| \\ \mathrm{OH}}}{\mathrm{C}}-$$

but rather as a mixture of the two in dynamic equilibrium with one another. Moreover, the chemical activity of this group as concerns either the nitrogen atom or CO group is determined entirely by this dynamic condition, for in a great number of cases it has been proved that a molecule in a static quiescence is singularly inactive to all chemical reagents. In order that a molecule should be reactive it is necessary that a certain amount of dynamic oscillation between the residual affinities be present, and the reactivity is proportional to the amount of dynamic isomerism which exists. In the case of the - NH - CO - grouping in question the phenomenon seems to be connected with the

1. Moore, Roaf, and Whitley, *Proc. Roy. Soc.*, B. 77, p. 102, etc., 1905. Moore and Wilson, *Bio-Chemical Journal*, Vol. I, p. 297, 1906. Moore, Roaf, and Knowles, *ibid.*, Vol. III, p. 279, 1908.

wandering of the hydrogen atom from the nitrogen to the oxygen. Now in all such cases the dynamic isomerism is increased by the addition of alkali and decreased by the addition of acid. Since the chemical reactivity of a substance depends upon the amount of dynamic isomerism present, so the reactivity of protein must be enhanced by the addition of alkali and depressed by the addition of acid. The activity of a cell must essentially be determined by the chemical reactivity of its components, so it is a natural sequence that alkali will stimulate and acid depress the normal functions of a cell. If we apply this argument to the special case already considered of a somatic cell whose chromatin granules vary very much in activity, it leads to an interesting conclusion. It was previously shown that owing to the potential gradient arising from the variation in the activity of the chromatin, a pseudo-reducing division can occur with production of cells possessing enhanced activity, or, in other words, a new growth is started. The application of alkali to a cell of the above type will stimulate the activity of all the granules present, and will consequently increase the potential gradient in the chromatin. The application of alkali would tend, therefore, to increase the probability of the pseudo-reducing division, that is to say alkali would tend to act as a direct cancer irritant when the necessary conditions are present, the necessary conditions being the existence of a gradation in the vitality or activity of the chromatin granules.

The term alkali has been used in the broad sense of any basic substance, and there seems no reason to doubt the direct connection between cancerous growth and irritation by basic substance. Two of the best known examples need only be quoted, namely, chimney-sweep's cancer, where the soot is the irritant, and, further, the undoubted connection between tobacco smoke and cancer of the lip and tongue. Both soot and the distillation products of tobacco are strongly alkaline substances, since they contain nitrogenous bases.

When once the new growth has started, the metabolism of the new cells comes into action, and it is not improbable that a chemical stimulus may arise from the hydrolytic and degradation products therefrom. It is conceivable that these products may themselves act upon the surrounding tissue cells and cause them to undergo the pseudo-reducing division. For this reason we do not wish to restrict ourselves to the statement that infiltration is due entirely to conjugation of the maximum charged cells with neutral tissue cells. The chemical stimulus arising from the hydrolytic and degradation products of the cancer cell metabolism can,

and doubtless does, disorganise the surrounding tissue cells, causing them to undergo the pseudo-reducing division, thus infecting them with the cancer taint.

The variations in the type of initial stimulus account readily enough for the various types of cancer which are known to arise in the same type of tissue. While it should not be possible from a given stimulus to develop more than one type of cancer cell, yet the possibility is by no means precluded of producing a new type of cancer in experimental transplantation. If a tumour be ingrafted on to a new host and if the new host were closely similar in every way to the first host, the new tumour would grow and infiltrate without great difficulty. A slight difference between the two hosts would tend to increase the resistance of the second to the ingrafted tumour, with the result it would become encapsuled. On the other hand, it should be possible to produce a new cancer infection by virtue of the chemical stimulus arising from the degradation products of the cell metabolism of the ingrafted tumour. This new infection might give rise to a cancer of the same type as the original tumour, but it might also, if slightly differently differentiated cells were affected by the chemical stimulus, give rise to a different type of new growth altogether.

Although the connection between cancer incidence, inbreeding, and hybridisation follows quite naturally from the theory of cytological processes advanced above, yet we feel the importance of entering more fully into the detail of a subject which, taken as a whole, would seem to open up a new field of research, namely Mendelism and environment as explained by electro-cytology. To turn to the major factor of the equation, in Mendelism we see the synthetical links which bind together the variations in chromatin distribution with racial index of cancer incidence. We have already pointed out that one of the fertile sources of cancer lies in the existence in the chromosomes of an individual of a certain number of chromatin granules of a poor or weakly type which tend to cause fusion of the chromosomes in mitosis with the production of daughter cells with a reduced number of chromosomes. The effect of this will be most marked when the segregation of these chromatin granules into one or other of the paternal or maternal sets of chromosomes occurs. However the Mendelian segregation of these granules takes place in the maturation division of the germ cells of the mothers, their ova will on the average contain chromosomes possessing a definite number of these granules. These ova, when fertilised by spermatozoa from men of

lower cancer incidence, will give rise to individuals having the same cancer incidence as the mothers, because the cancer incidence depends upon the presence of the weak granules in the one set (maternal) of chromosomes. A further reason for the maintenance of a mother's cancer incidence in her children is the fact that the cytoplasm of their cells is entirely derived from the maternal side. The first generation resulting from hybridisation, therefore, must preserve the cancer incidence of the mothers, although the fathers have a smaller incidence. The second generation of hybridisation will, however, decrease the cancer incidence, as can readily be seen. The cells of the first generation possess two sets of chromosomes: the maternal with their weakly granules and the paternal without them. When the meiotic gemini are formed and the De Vries re-arrangement occurs the weakly granules distribute themselves, and on the average the chromosomes formed by the splitting of the gemini will have fewer of the weakly granules. The children produced from these gametes with gametes of the new stock will of necessity have a lower cancer incidence. We have, therefore, a direct connection between the Mendelian segregation of the weaker chromatin granules and cancer incidence, a fact which explains the different incidence in the children of one family, and also the frequently observed skipping of a generation by the disease.

It would appear from what has already been said that cancer as a disease cannot be inherited: it is only the predisposition to the disease which is inherited, and we have shown that this predisposition must be influenced by inbreeding and hybridisation. We have emphasised the fact that for inbreeding to have any evil effects, it must occur throughout more than one generation; it is the inbreeding of a stock already inbred that will lead to deterioration.

Again, it is obvious that hybridisation in one generation cannot prevent the diathesis being handed down to the descendants, if the offspring of the first hybridisation be again inbred, for whether the predisposition in the inbred race is dominant or recessive to the hybrid, on again inbreeding there must be produced some pure dominants or recessives, as the case may be; on the other hand, every successive generation of hybridisation increases the immunity to the disease.

After consideration for some years past of the prevailing views concerning the aetiology of cancer, we are forced to conclude that the explanation is to be found in a study of cytology and cytopathology. We put forward in this paper the view that all malignant growths are due

to a derangement of the electrostatic forces normally present in somatic mitosis, which is initiated in the first place by a definite stimulus, internal or external, physical or chemical.

We have shown that the susceptibility of the cell to this derangement will be increased with decreasing vitality, such as occurs with age and as the result of inbreeding. Though fully cognizant of the fallacies inherent in statistics, we venture to refer to the results of a study made by us of the geographical distribution of cancer results which confirm the important rôle played by inbreeding, hybridisation, and racial immunity. We propose to publish these statistics in detail elsewhere, and will at this time only very briefly deal with the more important facts which have come to light.

Inasmuch as the highest cancer incidence known is to be found in Switzerland, we have paid special attention to this country, and have made an exhaustive investigation. The data have been corrected for age and sex constitution of the population, and the results obtained demonstrate clearly that it is in the isolated communities, which have been created by their geographical positions and the influence of religious antagonism, we meet with the highest cancer incidence, while in the passes that have served since the time of the Romans as the highways of invasion and commerce from Northern and Central Europe into Italy, we meet with the lowest.

Thus the cancer incidence (54·77 per 10,000 persons living, aged 30 and over) is highest in the canton of Appenzell in Rhoden, a Catholic canton, wherein until 1848 no Protestant or even Catholic aliens were allowed to settle. It falls to 27·70 in the neighbouring district of Ober-Rheintal, which lies in the valley of the Rhine, in the pass which leads directly to Chur, and from thence by the Splügen to Italy.

In the canton of Graubunden the lowest cancer incidences are in the passes, viz.:—Bernina (23·56), Munstherthal (24·01), and Maloja (22·87).

The cantons of Ticino and Valais have a remarkably low cancer incidence, 19·34 and 11·61 respectively. In Ticino those districts bordering on the St. Gothard Pass (Bellinzona 12·28 and Blenio 12·60) have the lowest incidence, which gradually rises to its maximum in districts most remote from the St. Gothard. The same occurs in Valais, and it is the district of Entremont (4·11) traversed by the Great St. Bernard Pass, which has the lowest cancer incidence for the whole of Switzerland.

The immunity produced by hybridisation is fully borne out by the low cancer incidence amongst the Eurasians, as reported by Dr.

Sutherland, of the Mayo Hospital, in the Third Report of the Cancer Research Laboratories of Middlesex Hospital (p. 87), wherein he says:—

> 'A striking fact is the small number of cases amongst Eurasians, who make up a large proportion of the in-patients in the Albert Victor wing of the Mayo Hospital. Only one case of carcinoma and one abdominal growth occurred out of 790 admissions for malignant disease.'

We have also made investigations as to the local origin of cancer, and we find, in comparing the cancer incidence in the various organs between the sexes and different races, that wherever any organ is specially liable to stress or excessive irritation, there is an increase in the number of malignant growths of that organ in the sex or race under consideration.

Although exception may be taken to any conclusions which are drawn from purely statistical data, yet it would appear that the evidence so arrived at is overwhelmingly in support of the fact that one of the major predisposing causes of cancer is to be found in close inbreeding. Moreover, it would also appear from a detailed comparison of the organs attacked, that those organs which are subjected to stresses and irritation are most liable to develop malignant growth. Both of these conclusions are in close agreement with the theory of electrocytology put forward in this paper.

Conclusions

The following conclusions are arrived at in this paper:—

1. The phenomena of somatic mitosis are readily susceptible of explanation by a simple theory of electrostatically charged colloids.

2. The simplest possible case is that when there are present in the cell hereditary characteristics of only one type, as exists in unicellular organisms.

3. When hereditary characteristics of two types occur, a reducing division is bound to occur at some period owing to the fusion of the chromosomes of opposite type. This reducing division is the forerunner of the maturation divisions of the more highly developed species.

4. The reducing division establishes residual charges of electricity in the daughter cells, the amount of charge depending upon the amount of differentiation between the opposite types of the characteristics.

5. In the animal kingdom where the sex differentiation is complete, the reducing division only occurs normally in the germ cells, and this

division is followed by one somatic division. After this second division no further divisions can take place owing to the magnitude of the residual charges developed.

6. In the lower types, such as occur in the vegetable kingdom where the sex differentiation is incomplete, the reducing division is not confined to the germ cells, but all the cells undergo it at some period of their development. The daughter cells of the reducing division give rise to an indefinite number of cells with the reduced number of chromosomes. Of these cells, in any one generation two have maximum residual charges of positive and negative electricity respectively, a fixed number are neutral and the remainder carry intermediate charges. The highest charged cells fuse together with restoration of the original number of chromosomes, thus completing the cycle. The occurrence of the reducing division endows the daughter cells with renewed vitality.

7. In the animal kingdom the four spermatozoa all carry different charges, one a charge equal to that of the ovum, one an equal and opposite charge, while the other two have intermediate charges.

8. The phenomenon of sex production may be attributed to these residual charges; all the phenomena of parthenogenesis, artificial fertilization and sterilization by X-rays are explained by the same theory.

9. The distribution of chromatin granules, required by the De Vries theory, is established as a necessary consequence of the maturation division.

10. The occurrence of pathological mitoses, as the result of external stimulus or internal stress, is established provided that the inherent precancerous condition be already present.

11. The possibility of a pseudo-reducing division of somatic cells is accounted for.

12. These pathological mitoses result in the establishment of residual charges in the daughter cells similar to those of the maturation division.

13. The daughter cells of the pseudo-reducing division possess renewed activity. They possess potential probability of conjugation with leucocytes and normal tissue cells.

14. The direct stimulation by alkali and bases generally is found to be a normal action, and it would seem that in certain cases alkali can act as a direct cancer irritant.

15. The stimulation of the surrounding tissue cells by the degradation products of the cancer cell metabolism is possible, and to this and the

facts mentioned in 13 is attributed the formation of a neoplasm with power of continuous growth.

16 The susceptibility of the cell to these derangements is increased with decreasing vitality, such as occurs with age, and as the result of in-breeding.

17. In close in-breeding through several generations the chromatin granules become more and more uniform so that they give in mitosis daughter particles of smaller and smaller potential difference, which markedly increases the tendency to abnormal mitosis; conversely hybridisation produces a maximum of cell stability and an individual with all its Mendelian allelomorphs as differentiated as possible.

18. The rate of proliferation depends upon the activity of the cytoplasm; the greater the activity the more rapid the growth, while the more highly differentiated the cell and the older it is the less rapid the rate of proliferation.

19. Age incidence, local origin, infiltration, metastases, transmission with all its limitations, and power of continuous growth are the natural outcome of abnormal cell proliferation induced by a disturbance of the electrostatic forces present in normal mitosis.

THE ESTIMATION OF PHOSPHORUS IN URINE

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As a preliminary to some investigations on the metabolism of Phosphorus, an examination of some of the methods that have been employed for the estimation of P_2O_5 in urine was carried out. In view of the probable presence of organic phosphorus compounds, Neumann's method, as modified by Plimmer and Bayliss (1), was also employed.

First Neumann's method, precipitation with magnesia mixture and precipitation with magnesium citrate mixture,[1] were compared on a solution of K_2HPO_4, and were found to give results concordant within one per cent. For Neumann's method 10 c.c. of urine are combusted with 10 c.c. of sulphuric acid, repeated small amounts of nitric acid being added towards the end of the combustion. The rest of the procedure is that described by Plimmer and Bayliss.

For inorganic phosphates, about 4 c.c. of the magnesium citrate mixture are added to 10 c.c. of urine, and enough ammonia to make the mixture smell distinctly of ammonia. The mixture is well stirred and let stand over night, and is then filtered through an ash free paper. The precipitate is dried and ashed; P_2O_5 is calculated from the ash, $Mg_2P_2O_7$.

Since the magnesia mixture method was found unreliable, it is not described in detail.

When the three methods mentioned were applied to urine, the highest values were given by Neumann's method, while magnesia mixture gave higher values than magnesium citrate. Neumann's method or magnesium

1. This reagent is prepared as follows:—Dissolve 40 grams of citric acid in 500 c.c. of water, add to the hot solution 20 grams of light magnesium oxide. Cool; add 400 c.c. of 0·880 ammonia and water to 1500 c.c. Let stand twelve to twenty-four hours, and then filter.

citrate gave practically constant results, but those given by magnesia mixture showed considerable variations. The P_2O_5 in the filtrates after precipitation with magnesia mixture or magnesium citrate was determined by Neumann's method.

TABLE I—COMPARISON OF NEUMANN, MAGNESIUM CITRATE AND MAGNESIA MIXTURE METHODS

Grams P_2O_5 in 100 c.c. Urine

	Neumann	Magnesium Citrate		Magnesia Mixture	
		Ppt.	Filt.	Ppt.	Filt.
I ...	0·153 0·156	0·144 0·143	— 0·011	0·151 0·156	0·005 0·004
II ...	0·242 0·238	0·223 0·223	— 0·013	0·238 —	0·004 —
III ...	0·224 0·230	0·204 0·207	0·017 0·019	0·228 0·242	0·006 0·012
IV ...	0·160 0·166	0·153 0·151	0·016 —	0·160 0·164	0·004 0·007

The sum of the magnesium citrate precipitate and filtrate values corresponds fairly with the Neumann value; but the sum of the magnesia mixture precipitate and filtrate values is considerably higher. This discrepancy will later be shown to be due to errors inherent in the magnesia mixture method.

The magnesium citrate results are very constant, even when quantities varying from 2 c.c. to 10 c.c. of the solution are used to 10 c.c. of urine. The precipitate is insoluble in 1 : 3 ammonia, for the results are not affected by prolonged and frequent washing.

To obtain evidence of complete precipitation of phosphates, Scott's reagent (2), which is capable of detecting 0·005 milligram inorganic P_2O_5, was applied to the filtrate after separation of the citrate precipitate. No reaction was obtained. It was found, however, that in the presence of citrates, Scott's reagent was far from delicate, so that the desired evidence had to be obtained in a less direct way, which will be described later.

The non-agreement between magnesium citrate and magnesia mixture values required some explanation. Neumann's method, applied to the ash from citrate precipitate, showed the theoretical amount of P_2O_5 to be present; applied to the ash from magnesia mixture precipitate, it showed less P_2O_5 than the theoretical, the amount being variable.

Table II—Estimation Gravimetrically and by Neumann's Method of P_2O_5 present in Ash from (i) Citrate, (ii) Magnesia Mixture Precipitates

Grams P_2O_5 in 100 c.c. Urine

	Magnesium Citrate		Magnesia Mixture	
	Gravimetric	Neumann	Gravimetric	Neumann
I ...	0·151	0·149	0·160	0·154
II ...	0·144	0·143	0·153	0·148
III ...	0·207 0·204	— 0·204	0·228 0·224	0·217 0·211
IV ...	0·194	—	0·270	0·211
V ...	0·226	0·222	0·235	0·227

If the magnesia mixture ash is dissolved in acetic acid, the addition of potassium oxalate shows the presence of calcium, which accounts for part of the error.

By adding magnesia mixture to the filtrate after precipitation with magnesium citrate, a further precipitate is obtained. This contains a little calcium, and also some phosphorus. The P_2O_5 was estimated in the ash gravimetrically and by Neumann's method.

Table III—Total P_2O_5, Inorganic P_2O_5 and P_2O_5 present in the 'Additional' Magnesia Mixture Precipitate

Grams P_2O_5 in 100 c.c. Urine

			'Additional' Ppt. with Mag. Mixture		
	Total	Inorganic	Gravimetric	Neumann	Residual P_2O_5 by difference
I ...	0·224 0·230	0·204 0·207	0·025 0·020	0·016 0·012	0·003 0·011
II ...	0·220 0·218	0·194 0·194	0·025 —	0·018 —	0·008 —
III ...	0·244 0·241	0·226 0·222	0·028 0·024	0·010 0·011	0·008 0·009
IV ...	0·222	0·203	0·014	0·006	0·013

This additional precipitation of P-containing substances suggested that magnesium citrate had failed to precipitate all the inorganic phosphates. Scott's reagent was applied to about 50 milligrams of the additional precipitate, and failed to give any reaction. Thus evidence was afforded of the complete precipitation of inorganic phosphates by magnesium citrate. The inaccuracy of the magnesia mixture method is thus due to two factors, first, that some calcium is precipitated; second, that some of the organic phosphorus is precipitated.

Ammoniacal solutions of barium chloride and calcium chloride have been used to precipitate phosphates. Since these reagents also precipitate sulphates, they are not convenient for quantitative estimations in urine. But in the filtrate from these the organic phosphorus of urine can readily be determined by Neumann's method. Indeed, this direct determination is much more readily performed in the barium chloride filtrate than in that from magnesium citrate, since the combustion of the latter is a matter of considerable difficulty.

TABLE IV COMPARISON OF ORGANIC P_2O_5 VALUES BY DIFFERENT METHODS

Grams P_2O_5 in 100 c.c. Urine

	Total (Neumann)	Inorganic (Citrate)	Organic: Total, minus Inorganic	Organic: Citrate Filtrate	Organic: $BaCl_2$ Filtrate
I ...	0·242	0·222	0·020	0·018	0·016
II ...	0·156	0·143	0·013	0·011	0·011
III ...	0·142	0·127	0·015	0·012	0·013
IV ...	0·180	0·170	0·009	0·009	0·011
V ...	0·196	0·183	0·010	0·015	0·011

The substantial agreement of these methods affords further evidence of the accuracy of the citrate method for inorganic phosphates. The difference between Neumann and magnesium citrate values represents the amount of phosphorus present in organic combination.

The uranium acetate method was found useless for accurate work.

Répiton (3) has shown that uranium acetate solutions must be standardized for the particular phosphate which is being estimated. Thus the method is inaccurate in a solution of mixed phosphates.

TABLE V—COMPARISON OF NEUMANN, MAGNESIUM CITRATE AND URANIUM ACETATE[1] VALUES

Tincture of Cochineal as a warning and Potassium Ferrocyanide as final indicator.

Grams P_2O_5 in 100 c.c. Urine

	Neumann	Mag. Cit.	Uran. Acet.
I ...	0·229 0·233	— 0·205	0·213 0·217
II ...	0·201 —	0·187 —	0·198 0·191
III ...	0·166	0·156	0·166
IV ...	0·204 —	0·194 —	0·208 0·198
V ...	0·196 0·198	0·183 —	0·192 0·186
VI ...	0·154	0·142	0·160

1. This uranium acetate was standardized against the K_2HPO_4 solution originally employed to test the different methods.

The uranium acetate values are usually somewhere between the total and the inorganic values, but may be above the total. Any attempts to determine organic P_2O_5 by methods involving the use of uranium acetate must give incorrect results.

The Disappearance of Organic Phosphorus

In the course of the investigation it was several times noticed that duplicate samples left some days before being analysed, gave lower values for organic phosphorus than samples analysed immediately; in some cases no organic phosphorus was found. It was thought that this might be due to the destruction of organic phosphorus compounds, perhaps by the enzymes present in the urine.

Samples of urine were kept in aseptic flasks, in an incubator, at 37° C., a little toluol being added. Small quantities were removed at intervals of a few days, care being taken to cool the flask so as to obtain the correct volume, and estimations of inorganic P_2O_5 made. In other cases ammonia was added to the urine, which was similarly incubated. Owing to the precipitation of phosphates it was impossible to obtain uniform samples, so the organic P_2O_5 was estimated in the filtrate from 50 c.c. of urine after treatment with magnesium citrate.

The following are a few of the results:—

I	Jan. 12.	250 c.c. Urine + toluol at 37° C. Total P_2O_5 = 0·196 grams in 100 c.c. Urine
		Inorganic P_2O_5 = 0·183 „ „
	„ 15.	Inorganic P_2O_5 = 0·187 grams in 100 c.c. Urine
	„ 22.	„ „ = 0·194 „ „
	„ 29.	„ „ = 0·194. „ „ No phosphorus present in filtrate
II	Feb. 13.	250 c.c. Urine + toluol, at 37° C. Total P_2O_5 = 0·247 grams in 100 c.c. Urine
		Inorganic P_2O_5 = 0·230 „ „
	Feb. 27.	Organic P_2O_5 = 0·009 grams in 100 c.c. Urine
	Mar. 25.	„ „ = 0·007 „ „
III	Feb. 13.	250 c.c. Urine + 10 c.c. Ammonia at 37° C.
	„	Total P_2O_5 = 0·282 grams
	„	Inorganic „ = 0·255 „
	Feb. 20.	Organic P_2O_5 = 0·007 „
	Feb. 27.	No trace of Organic P_2O_5 present

These results show that the organic phosphorus compounds are partially or completely broken down when urine is kept at body temperature. This decomposition is accelerated by ammonia. It is, therefore, important not to leave urine standing too long after adding the magnesium citrate mixture, and also to make estimations of organic P_2O_5 in fresh urine.

As it has been suggested, though without adequate proof, that glycerophosphoric acid is the form in which organic phosphorus is present in urine (4), 10 c.c., containing 1·2 grams organic P_2O_5 were neutralized and added to urine containing a known amount of organic P_2O_5. The urine was left in the incubator for a month. The increase of inorganic phosphates was no greater than could be accounted for by the breaking down of the pre-existing organic phosphorus compounds; the glycerophosphoric acid escaped destruction.

The Results of Dialysis

It was thought that by dialysis it might be possible to remove the inorganic phosphates and leave organic phosphorus behind. Urine in quantities of 200 c.c. was dialysed for some days, the dialysing fluid being changed three times a day. In some cases continuous dialysation was employed during the last twenty-four hours. Nearly all the phosphorus dialysed out in the first twenty-four hours; at the end of four days no phosphorus could be detected in the residual fluid. By the addition of Folin's ammonium sulphate and uranium acetate reagent to this fluid a nitrogen-containing substance was precipitated, but it contained no phosphorus. Filtrates obtained after precipitating urine with magnesium citrate were similarly dialysed, but no phosphorus-containing substances remained in the tube. The addition of Hedin's tannic acid reagent to urine occasionally produced a slight precipitate, but no phosphorus could be found in this. The organic phosphorus compound of urine is thus not identical with the protein-like material sometimes present.

Summary

1. The estimation of total phosphorus in urine is most conveniently carried out by Neumann's method as modified by Plimmer and Bayliss.

2. Inorganic P_2O_5 is best estimated by precipitation with magnesium citrate mixture, incineration and calculation from the weight of the ash, $Mg_2P_2O_7$. This method is shown to precipitate inorganic phosphates completely.

3. Magnesia mixture gives incorrect and variable results, partly owing to the precipitation of calcium, partly to precipitation of a portion of the organic phosphorus.

4. Organic P_2O_5 can be determined either by subtracting the inorganic from the total P_2O_5, or directly by applying Neumann's method to the filtrate after precipitation of inorganic phosphates by magnesium citrate or barium chloride.

5. The uranium acetate method is unsuitable for accurate work. Statements as to the existence or non-existence of organic phosphorus in the urine based on uranium acetate estimations are valueless.

7. It is important to make the determinations of organic P_2O_5 in fresh samples of urine, as the organic compound is partially or completely decomposed in the course of a few weeks, or, if ammonia be present, in a few days.

8. The organic phosphorus compound is readily dialysable, and is not precipitated by reagents that precipitate traces of protein.

It is with great pleasure that I acknowledge my indebtedness to Dr. Aders Plimmer for his freely given help and suggestions as to methods in this investigation.

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ON THE NITROGEN-CONTAINING RADICLE OF LECITHIN AND OTHER PHOSPHATIDES

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PART II

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In a former article[1] it was shown that only about 42 per cent. of the nitrogen of heart muscle lecithin could be accounted for when estimated in the form of the choline-platinum-chloride salt. When a sample of trade lecithin was treated in a similar manner it was found that the yield was considerably higher, being equivalent on an average to about 76 per cent. of the total nitrogen of the substance.

The comparatively low choline content of heart lecithin suggested the probability of part of the nitrogen being represented by some other N-complex differing from choline in its precipitation properties. As the method of preparation of the trade lecithin could not with certainty be ascertained, lecithin was prepared from egg yolk; an analysis of this lecithin agreed in general with that obtained from the heart, but, as will be shown below, the amount of nitrogen that could be accounted for as above described was considerably greater than in heart lecithin, and a good deal lower than in trade lecithin.

This curious result seemed to point to the probability of these different lecithins being really different bodies, at least with regard to the manner of combination of their nitrogen, and in order to test this a long series of experiments was made.

In this paper it is proposed to deal with the results of hydrolysis of egg lecithin, and at the same time describe certain experiments made with a view to ascertaining whether possibly any circumstances were present that might reduce the final output of choline, even assuming that all the nitrogen was present as this base, in accordance with the ordinary formula for lecithin.

PREPARATION OF EGG LECITHIN

Lecithin was prepared from three different portions of eggs. The method adopted was essentially the same as that described in my former papers,[2] and need not be repeated; in every case (with one exception)

1. This Journal, Vol. IV, p. 38.
2. This Journal, Vol. IV, pp. 47 and 168.

I used the lecithin obtained from the *ethereal* extract of egg yolk, the usual precautions being adopted to exclude, as far as possible, air and light during the process of preparation.

In one set of eggs a curious state of affairs was noticed; these eggs (100) seemed to be perfectly fresh and were all rather large, it being naturally thought that this would ensure a greater yield of lecithin. On extraction, however, it was found that the ether showed quite an abnormal increase of fatty matter, but only a trifling amount of substance precipitated by acetone; after treatment with five consecutive portions of fresh ether, the extract still contained much fat, and the combined yield of lecithin was so small that after purification the total amounted only to a few grammes. On subsequent extraction with alcohol the lecithin yield was also very much below the ordinary average.

In this particular case it would seem as if there was a great increase of fatty matter at the expense of the lecithin; unfortunately, I had not an opportunity of further investigating the nature of this substance, but a comparison of the relationship of fat to lecithin in the egg, and perhaps in other organs, together with an examination into the nature of this fat, would, in the light of the above observation, probably be of some interest.

A curious point with regard to the lecithin here obtained was that it differed greatly in general appearance from lecithin obtained from other eggs; it was from the beginning quite dark brown in appearance and not so plastic as is generally the case, despite the fact that the greatest precautions were taken to prevent oxidation; in general it looked more like a specimen that had been exposed for some time to air than the freshly extracted material, which is usually precipitated as a plastic, more or less whitish mass with a slight brown tinge. The amount of lecithin here obtained was so small that it was not made use of for this investigation.

The first sample purified gave the following figures on analysis: comparison with heart muscle lecithin shows a marked similarity in elementary composition:—

			Egg Lecithin	Heart Lecithin
N	(3 experiments)	Average	1·876 %	1·87 %
P	2 ,,	,,	3·95 %	3·95 %
C	1 experiment	,,	64·18 %	66·29 %
H	1 ,,	,,	10·6 %	10·17 %

N : P = 1·05 : 1

Hydrolysis of Lecithin

(*a*) *In alcoholic solution of barium hydrate.*—Here about 1 gramme lecithin was boiled for varying periods of from two to six hours with 100 c.c. methyl alcohol containing 5 grammes $Ba(OH)_2$. After separating certain products of decomposition, as formerly described, the purified alcoholic extract was treated with alcoholic-platinum-chloride solution, and the precipitate washed, dried and weighed as usual.

Two experiments taken at random from a long series gave the following results; percentages are expressed in terms of actual amount obtained, as against theoretical amount calculated on N present:—

1·0268 gm.	Lecithin boiled	3 hours	= 0·2766 gm.	Choline-platinum-chloride	= 65·4 %
0·8989 ,,	,,	6 ,,	= 0·2448 ,,	,, ,,	= 66 %

Here, as in experiments on heart lecithin, it was invariably found that the residue obtained after hydrolysis contained nitrogen, despite the most prolonged and careful washing; experiments described later on suggest that this nitrogen is probably not of choline nature; in the two experiments given above, this insoluble N amounted to 6·08 per cent. and 9·13 per cent. respectively of the total nitrogen contained in the amount of lecithin used. In six experiments in which this residual nitrogen was estimated, the average percentage of the total lecithin nitrogen found in the residue was 6·74 per cent.

(*b*) *In watery solution of* $Ba(OH)_2$. In this case the hydrolysis was carried out in general as above described, certain modifications being adopted in order to obtain the end alcoholic solution of choline as free as possible from impurities. The time during which boiling was continued varied from three to five hours, but in one experiment this was extended to eighteen hours. A reference to the result shows that this prolonged heating had a comparatively trifling effect in destroying choline, and thereby lowering the percentage figures. In general, the results stand in accord with those obtained by the use of alcoholic solution; the following three experiments suffice to show this:—

0·8403 gm.	Lecithin boiled	3 hours	= 0·2246 gm.	Choline-platinum-chloride	= 64·8 %
0·7821 ,,	,,	5 ,,	= 0·2103 ,,	,, ,,	= 65·2 %
0·7411 ,,	,,	18 ,,	= 0·1920 ,,	,, ,,	= 63 %

Thus, it is seen that this specimen of lecithin when hydrolysed in a watery or alcoholic solution of $Ba(OH)_2$ gave practically similar results, varying on an average from 65 to 66 per cent. of the theoretical amount. Here, as with alcohol, the residue obtained after filtration of the hydrolysed solution invariably contained some nitrogen.

In a few experiments a special attempt was made to render this residue free from nitrogen, but without avail. After filtration the substance was thoroughly broken down, returned to the flask, and boiled for fifteen minutes with 50 to 80 c.c. H_2O. Again it was broken down and treated as before, this process being repeated three times; afterwards it was carefully washed on the filter with water almost at boiling temperature. Despite this prolonged washing, examination in every case revealed the presence of a distinct amount of nitrogen in the residue.

(*c*) *In mineral acid solution.*—In almost all the text-books, both old and modern, there appears the statement that lecithin while easily split up by the action of an alkali, is but very slowly attacked by acids. In testing this, however, it was found that such is not the case, at any rate with regard to the splitting off of choline. After boiling with a weak acid for a comparatively short time, choline seemed to be completely separated off, and as the use of acid in this connection possessed certain advantages some experiments were carried out with it. At first sulphuric acid was used, but the difficulties experienced in getting rid of it after hydrolysis was completed rendered the method somewhat cumbersome, and instead of sulphuric acid use was made of hydrochloric acid. Here it was thought that removal of the acid previous to precipitation by platinum chloride would not be necessary, though after evaporation of the watery part of the solution it was obviously present in fair concentration, and some experiments (described later) showed that this assumption was correct.

In all these hydrolytic processes it is an advantage to avoid if possible the use of barium, owing to the great difficulty of getting rid of it completely afterwards. With an alcoholic solution this is a matter of very great difficulty, and even with water, from which separation is much more easily obtained, it is sometimes found to be present in traces towards the end of the operation of purifying. The danger in the presence of barium results from the fact that this substance forms a double salt with platinum chloride, which may be thrown down with choline-platinum-chloride. It is true that this barium salt is fairly easily soluble in alcohol, but in spite of this fact, great care must be taken to wash the platinum chloride precipitate thoroughly with cold alcohol when there is any chance of barium being present. If this is not attended to somewhat variable results, difficult to account for, may be obtained. In all my experiments in which $Ba(OH)_2$ was used, particular care was taken to ensure the thorough washing of the platinum chloride precipitate in order to dissolve out any traces of the double barium salt that might be present.

When this precaution is neglected, it is quite possible that traces of barium may sometimes account for the apparent percentage of platinum being somewhat above the theoretical amount when the salt of choline-platinum-chloride is ignited.

With HCl, however, the separation of barium is got rid of, and, so far, a great simplification introduced. Experiment showed that all that is necessary is to boil the lecithin for some time with a weak HCl solution, filter, evaporate to dryness, dissolve in absolute alcohol and precipitate directly with platinum chloride. This method takes up very much less time than when done with $Ba(OH)_2$, and is to be recommended when estimating the choline of lecithin; here the choline is present from the beginning in the form of the chloride compound, and as such is less likely to undergo decomposition than when present in the free state in alkaline solution.

As pointed out above, however, the action of $Ba(OH)_2$ on choline in saturated watery or alcoholic solution is not of much importance, the loss in a sample boiled for eighteen hours with 5 per cent. $Ba(OH)_2$ in H_2O amounting only to about 2 per cent. of the total choline present. In my experiments the HCl hydrolysis was performed as follows:—

About 1 gramme substance was taken and boiled for varying periods with 100 c.c. of a 10 per cent. watery solution of hydrochloric acid (10 c.c. HCl sp. gr. 1·81 + 90 c.c. H_2O), boiling being continued with the aid of a reflux condenser for from two to five hours. The fatty material separated out as an oily mixture, and could not be conveniently filtered off while the solution was hot, but on cooling formed a solid mass, which was easily separated from the liquid. This fatty residue was returned to the boiling flask, and again boiled with 100 c.c. H_2O for ten to fifteen minutes; the mixture was then allowed to cool and filtered as before, the process being in many cases repeated three times. The total filtrates mixed together were now evaporated to dryness on the water bath, and the residue extracted with absolute alcohol, filtered, evaporated to small bulk and precipitated directly with platinum chloride. It was then left to stand till next day, and the precipitate then filtered, washed, dried and weighed as usual.

The obvious object of the above prolonged washing of the fatty residue was, if possible, to render it nitrogen free. This, however, could not be accomplished, and it was found quite unnecessary to return the residue to the flask more than once, as further washing did not lessen the nitrogen content.

It is noteworthy that in whatever manner lecithin is split up the residue always contains a certain amount of nitrogen, and since this residue is composed practically of fatty matter, it suggests the probability of this nitrogen being in close relationship with the fatty acid radicle of the lecithin body.

The following is an example of the figures obtained with HCl:—

0·7601 gm.	Lecithin	boiled	$1\frac{1}{4}$ hours	= 0·2012 gm.	Choline-platinum-chloride		= 64·2 %
0·8274 „	„	„	3 „	= 0·2222 „	„	„	= 65·1 %
0·9124 „	„	„	5 „	= 0·2425 „	„	„	= 64·5 %

The results are practically identical with those obtained when barium hydrate was used, and other samples of egg lecithin gave almost similar results. One sample, however, differed somewhat from the above in its nitrogen percentage,[1] and in this case a slightly larger yield of choline was obtained when calculated on the total nitrogen present, though when calculated on the weight of substance it was practically in agreement with the above sample.

A trade lecithin, having a nitrogen percentage of 1·77 and a N : P ratio of almost exactly 1 : 1, was now taken, and samples treated alongside of the egg lecithin as follows:—About 1 gramme of this lecithin and a similar amount of egg lecithin were hydrolysed under exactly similar conditions as parallel experiments; they were then treated as nearly as possible in the same manner, the same amount of fluid being used to wash the residue, &c., and the choline precipitated in the usual way.

One experiment was done using alcoholic $Ba(OH)_2$, another with watery $Ba(OH)_2$, and a third with HCl. The following results were obtained, and clearly prove that the two lecithins, though having a fairly similar elementary composition, are certainly not identical substances with regard to their nitrogen complex:—

Fluid used for hydrolysis	Percentage of choline of egg lecithin found	Percentage of choline of trade lecithin found	Difference in percentage
Alcoholic $Ba(OH)_2$	65·8	80	14·2
Watery $Ba(OH)_2$	64·6	79·6	15
„ HCl	65·3	80	14·7

Again, heart lecithin differs from these results in a greater degree than they differ from each other; a comparison of the three substances gives the following figures with regard to choline recovered as the double platinum salt:—

Heart lecithin = 42 %
Egg lecithin = 65 %
Trade lecithin = 80 %

1. This sample gave an average of 1·77 % N.

From these results it is obvious that so-called lecithins are really bodies of different composition, despite their general agreement when viewed from the results of elementary analyses.

Relation of N of End Filtrate to Choline-Platinum-Chloride actually obtained

As in the case of heart muscle lecithin, some experiments were done in order to test directly what proportion of the nitrogen in the end filtrate —i.e., the end alcoholic solution obtained after hydrolysis, and purified as much as possible from other decomposition products and ready for precipitation with platinum chloride—could be obtained as the double platinum salt. In some respects this direct estimation gives more definite results than the ordinary lecithin estimations described above, for it is known exactly what amount of the nitrogen appearing in the end solution is actually precipitated as choline. To determine this, hydrolysis was performed as before, and the alcoholic solution ultimately obtained divided into two equal parts. One part was used for N estimation, and the other precipitated directly with platinum chloride. In three experiments it was found that 23 per cent., 24 per cent. and 26 per cent., respectively, of the N actually present was not recoverable as choline-platinum-chloride.

All these results prove beyond any doubt that the nitrogen of the end filtrate is not present as choline, and suggests strongly that in lecithin the generally accepted formula is insufficient for the facts obtained.

In the light of the above results, two considerations present themselves—

(1) That the nitrogen of the lecithin is really not all present in the form of choline or similar basic compound; or

(2) That all the nitrogen is really present as choline, but that some interaction with the chemicals employed during hydrolysis, or with some of the substances of hydrolytic decomposition, gives rise to some unknown nitregenous complex which is not precipitated by platinum chloride.

In order, therefore, to strengthen the probability that lecithin N is not all present in the form of choline, it was necessary to thoroughly investigate this point; for this purpose pure choline chloride was used, and the following experiments performed:—

Experiments with Choline Chloride

Samples of the choline-platinum-chloride salt obtained in the above experiments were mixed together and dissolved in water, filtered and re-crystallised. The typical crystals obtained were again dissolved in water, and the process of re-crystallisation twice repeated. On ignition practically the theoretical amount of platinum was obtained. A strong watery solution of the crystals was then treated with H_2S to remove the platinum, the fluid being heated during the passage of the gas in order to ensure complete separation. The mixture was then freed from platinum sulphide by filtration, and the filtrate, which was water clear, evaporated to dryness on the water bath. Residue was then dissolved in absolute alcohol, and this solution used in the experiments.

The object of these experiments was to find out what percentage of the choline chloride actually present in the alcoholic solution could be recovered as the double platinum salt after the solution had been treated with $Ba(OH)_2$ in alcoholic, and in watery solution, and with HCl, exactly as in the hydrolysis of lecithin.

For each experiment an equal amount of the above solution was taken, and the following tests performed:—

A. For Nitrogen

5 c.c. choline solution was directly run in to a Kjeldahl flask and the N-content estimated with the following results:—

(1) Gave 9·24 mgr. N equivalent to 0·2031 grm. Choline-platinum-chloride
(2) ,, 9·24 ,, ,, ,, 0·2031 ,, ,, ,,

Here in both experiments the results were absolutely identical.

B. For Choline-platinum-chloride by Direct Precipitation

(1) 5 c.c. directly precipitated by 10 % alcoholic platinum chloride solution, allowed to stand 18 hours, filtered, dried and weighed:—

Result = 0·1961 grm. Choline-platinum-chloride

(2) Same as above, but 5 c.c. evaporated to about 2 c.c. before precipitated with platinum chloride:—

Result = 0·1968 gm. Choline-platinum-chloride

From the above it is seen that the amount of choline actually present in the solution was, on the nitrogen calculation, equivalent to 0·2031 gramme of the double platinum salt, and calculated on direct precipitation about 0·1964 gramme, taking the average of the two experiments. This slight difference may be accounted for by the difficulty of absolute exactness in allowing for the necessary reduction of nitrogen formed from

the chemicals used in the Kjeldahl estimation, coupled with the ordinary experimental errors and perhaps a slight loss due to traces of the choline remaining in solution. It would seem that evaporation of the choline containing fluid to very small bulk is not necessary, as the above results with volumes of 5 c.c. and 2 c.c., respectively, are practically the same. As a result of all these figures, it is clear that 5 c.c. of this solution ought to yield at least 0·1960 gramme of the double salt when precipitated by platinum chloride.

C. *Choline Solution Boiled with Different Reagents to Imitate Hydrolysis of Lecithin*

(1) *Watery solution of $Ba(OH)_2$.*—To 100 c.c. of a 5 per cent. solution of $Ba(OH)_2$ in water, 5 c.c. of choline solution was added and the mixture boiled for three hours, a reflux condenser being used. After cooling the solution was filtered, the filter paper thoroughly washed, and the combined filtrates treated with CO_2 to precipitate the barium. Mixture was now filtered and the barium carbonate residue thoroughly washed with hot water. Filtrates were united, a few drops HCl added, and evaporated to dryness. Residue was then dissolved in alcohol, evaporated to small bulk and precipitated as before.

Gave 0·1963 gm. Choline-platinum-chloride
,, 0·1959 ,, ,, ,,

(2) *Methylic alcohol solution of $Ba(OH)_2$.* This was carried out as above, with a few modifications mentioned in a former article, to ensure the removal of the barium. Owing to an accident, only one experiment was completed; it gave—

0·1957 gm. Choline-platinum-chloride

(3) *Hydrochloric acid.*—(*a*) To 100 c.c. of a 10 per cent. watery solution of HCl, 5 c.c. choline chloride was added and the mixture boiled for two hours; it was then filtered and the filter paper thoroughly washed. Filtrate was then evaporated to dryness over the water bath, residue dissolved in absolute alcohol, filtered, evaporated to a few c.c.'s and precipitated as usual.

(*b*) Here a 5 per cent. HCl solution was used, and boiling was continued for one and a half hours; otherwise it was identical with above.

(*a*) Gave 0·1970 g.m. Choline-platinum-chloride
(*b*) ,, 0·1951 ,, ,, ,,

It is thus obvious that HCl when used as above does not interfere to any extent with choline chloride. The only point in the manipulation at which some action might be expected is during evaporation to dryness of the dilute acid solution, for it is obvious that towards the end the concentration of HCl is very great; however, this apparently exerts no destructive action.

The following gives an indication in tabular form of the results obtained:—

CHOLINE-PLATINUM-CHLORIDE IN GRAMMES

Calculated from N found	Found by direct precipitation	Found, using HCl	Found, using alcoholic $Ba(OH)_2$	Found, using watery $Ba(OH)_2$
0·2031	0·1961	0·1970	0·1957	0·1963
0·2031	0·1968	0·1951	—	0·1959

The above experiments were carried out with every possible precaution in order to ensure parallel results, and a comparison of the figures shows that the greatest difference between any two amounts only to 0·008 gramme, the average difference being very slight indeed. When it is considered that a rather long process of manipulation, including several filtrations and the necessity of changing from vessel to vessel (evaporation, &c.), was involved the results agree remarkably well, and show conclusively that the loss of choline-platinum-chloride in the experiments on lecithin is not due to any destructive action on the part of the hydrolysing agents; further, they indicate that the necessary manipulations can be conducted with little or no loss, though in doing this the greatest care is necessary.

In view of the above results there remained only the possibility, already mentioned, that some inter-action between choline and some other product of lecithin decomposition might ensue, and so yield a nitrogenous complex of obscure nature which was not precipitated by platinum chloride. In order to test this a similar solution to the above was used, but to the $Ba(OH)_2$ or acid mixture some of the known products of lecithin decomposition were added before boiling. From the results obtained it is obvious that no such inter-action takes place. The experiments were carried out as follows:—

Experiments with Lecithin Decomposition Products

For these experiments a solution of choline chloride similar to above was used, but of somewhat different strength; 5 c.c. gave on direct precipitation 0·2200 gramme of the double platinum salt.

(*a*) 5 c.c. of this solution was added to 100 c.c. water containing 2 c.c. glycerophosphoric acid. This mixture was boiled for half an hour and 5 grammes $Ba(OH)_2$ added; boiling was continued for two hours, and after cooling a whitish residue was obtained on filtration. This residue was thoroughly washed three times with 100 c.c. boiling water, being each time returned to the flask and boiled for ten minutes; finally it was washed with hot water on the filter. Filtrates were then treated with CO_2, and the usual manipulations performed.

(*b*) This experiment was conducted exactly as above, only that $Ba(OH)_2$ was present at the beginning of boiling. Results were as follows:

(*a*) Gave 0·2173 gm. Choline-platinum-chloride
(*b*) " 0·2161 " " "

A number of experiments were now made with different amounts of other known decomposition products, such as glycerine, phosphoric acid, oleic acid, &c. The average results obtained showed that only a trifling loss was accounted for by the presence of these materials, and as their introduction gave rise to marked difficulties in the way of filtration and washing of residues, this small loss cannot be held to account for the great loss in lecithin experiments; in any case this loss did not amount to more than 6 per cent. of the total, and since much greater quantities of above products than would ever be obtained as the result of the hydrolytic decomposition of lecithin containing a similar amount of choline (equivalent to 0·2200 gramme platinum salt) were used, much more difficulty was experienced in filtering than is the case with lecithin.

The following experiment is sufficient to show that, even when excessive quantities of substances representing lecithin decomposition products are used, the final yield of lecithin is not materially decreased.

5 c.c. choline solution was mixed with the following substances:—

Glycerine	1 c.c.
Glycerophosphoric Acid	1 c.c.
Phosphoric Acid	0·5 grm.
Stearic Acid	0·5 grm.
Oleic Acid	0·5 c.c.

and the mixture boiled in a 5 per cent. watery solution of $Ba(OH)_2$ for four hours; it was then treated in the ordinary way to remove impurities. In this case the difficulties in filtration were very great, the experiment taking a very long time owing to the fatty nature of the residues obtained. The final yield, however, gave 0·2110 gramme choline-platinum-chloride, or about 95·5 per cent. of the total obtained on direct precipitation, showing definitely that when choline is really present as such at the beginning, such treatment does not materially lessen the final amount obtained.

Here again it is seen that the residue obtained on boiling choline with $Ba(OH)_2$ and other substances such as the above products gives a residue which *can* be rendered N-free. From this it must be assumed that the nitrogen remaining in the first and other residues in the hydrolysis of lecithin does really not represent nitrogen of choline, but is present in the form of another nitrogen complex.

The result of all the above experiments seems to show definitely that if choline were really present in lecithin to the amount represented by the nitrogen content, a much greater yield would necessarily be obtained on hydrolysis and precipitation with platinum chloride.

Since little or no evidence of the breaking down of choline can be obtained experimentally under the conditions present in the hydrolysis of lecithin, it is but fair to assume that a similar state of matters holds good for lecithin itself, and that the results actually obtained gave a very fair indication (allowance being made for slight losses due to possible defects of the method) of the amount of choline actually present as such in the original substance. A consideration of these facts shows the absolute futility of endeavours made at one time to estimate the amount of lecithin present in an organ in terms of the yield of choline obtained. It is obvious that this method would give widely divergent results when applied to, say, heart lecithin and egg lecithin respectively.

Thus the accepted formula for lecithin does not account for the facts, and in view of the differences existing between apparently similar lecithins but derived from different sources, can hardly be taken as a representation of any lecithin as obtained by the best methods at our disposal at present.

It was next thought that an investigation of some of the salts of lecithin such as the cadmium chloride compound might yield some information; the results obtained strengthen the above view.

Cadmium-Chloride-Lecithin

32·53 grammes lecithin were dissolved in absolute alcohol, and to this an alcoholic solution of cadmium chloride was added till no more precipitation occurred. After standing for eighteen hours precipitate was filtered off and thoroughly washed with cold alcohol, filtrate and wash alcohol being preserved. It was then dried and weighed, and yielded 36·41 grammes lecithin-cadmium-chloride.

Filtrate and wash alcohol together was now carefully evaporated to dryness and gave a total residue of 2·64 grammes, part of this obviously consisting of cadmium chloride.

This residue (which may be termed Residue A) was now treated with water in order to dissolve out the cadmium chloride, and was extracted till the solution gave no evidence of the presence of cadmium on the passage of H_2S.

On evaporating down this wash water it was tested and found to contain a good deal of nitrogen. A mere trace of phosphorus could be detected, the relation of N : P standing as 14·1 : 1.

Here it was obvious that some nitrogen must have been split off from the lecithin; on boiling this watery solution with HCl and treating in the usual way with platinum chloride, no precipitate could be obtained, indicating that this nitrogen was not present in the form of choline.

After this extraction Residue A weighed only 1·16 grammes, so that 1·48 grammes must have gone into solution, the greater part of this being cadmium chloride. Thus, the total amount of substance obtained from 32·53 grammes lecithin after removal of excess of cadmium chloride was 36·41 grammes + 1·16 grammes = 37·57 grammes. The remaining portion (1·16 grammes) was now thoroughly dried and analysed, with the following results:—

$$\text{Nitrogen} \begin{cases} 1{\cdot}73\ \% \\ 1{\cdot}77\ \% \end{cases} \text{average } 1{\cdot}75\ \%$$

Phosphorus 2·18 %

On treating in the usual way for choline, only about 20 per cent. of the theoretical yield was obtained, but the small amount of substance rendered it difficult to get quite an accurate result.

Since it is well known that lecithin is not entirely precipitated out of alcoholic solution by cadmium chloride, this residue might be expected to be composed of lecithin-cadmium-chloride; the analysis shows plainly,

however, that something more containing a higher percentage of nitrogen and a lower percentage of phosphorous than this compound must have been present as well. The choline-platinum-chloride found probably represents the choline of the double lecithin salt, while the remaining nitrogen was present in some other form. All these points go to strengthen the conclusion hitherto suggested, that the nitrogen of lecithin is not all represented by choline or such basic body.

Analysis of Lecithin-Cadmium-Chloride

In the light of the above results it was of interest to ascertain what percentage of the nitrogen in the double cadmium salt could be recovered in the form of choline-platinum-chloride. Since, in the manipulation described, nitrogen was found which did not seem to represent choline nitrogen, it was naturally concluded that the percentage of nitrogen actually representing choline in the double cadmium salt ought to be somewhat higher than in lecithin. This was found to be the case.

The following figures for N and P were obtained.

Nitrogen	1·414 % 1·416 %	average 1·415 %
Phosphorus	3·09 % 3·10 %	average 3·095 %

N : P = 1 : 1

From these figures it is, of course, easy to deduce the theoretical yield of cadmium-chloride-lecithin that should be obtained from a given quantity of lecithin. In the above sample the average nitrogen content of the lecithin used was 1·876 per cent., and of the cadmium salt 1·45 per cent. If all the nitrogen of the lecithin were contained in the cadmium salt a simple calculation shows that 1 gramme lecithin contains sufficient nitrogen to yield 1·294 grammes of the cadmium salt, and, therefore, the quantity used (32·53 grammes lecithin) ought to furnish 42·093 grammes lecithin-cadmium-chloride. The amount actually obtained after removal of excess cadmium chloride was, as mentioned, only 37·57 grammes—another proof that all the nitrogen present in the lecithin did not go to form the double cadmium salt.

Although, as shown, a certain amount of the lecithin-cadmium-chloride remained in solution, this was much too small to account for the loss of about 4·5 grammes calculated on the theoretical amount, using nitrogen as a basis.

Hydrolysis of Lecithin-Cadmium-Chloride

The salt was treated with watery solution of $Ba(OH)_2$ in the usual way, and the choline calculated as choline-platinum-chloride with the following results:—

0·6348 gm. Salt	= 0·1470 gm. Choline-platinum-chloride		= 74·5 %
0·5587 „ „	= 0·1777 „ „ „		= 75 %

These figures show that the cadmium salt of lecithin yields about 10 per cent more choline-platinum-chloride than lecithin itself does, results being in both cases calculated on the original N-percentage of the substance. This again shows that some nitrogen, which was not present as choline, must have been thrown off from the lecithin, otherwise the results cannot be explained. On the other hand, it is obvious that this lecithin salt must, like lecithin itself, contain a good deal of nitrogen which is not present as choline.

It is intended to again recover this lecithin from the salt, and to estimate its choline content; then to repeat the process of precipitation and analysis in order to ascertain whether this splitting off of nitrogen would be in evidence on a second precipitation.

It is quite possible that this particular part of the nitrogen may be present as an impurity in the form of some other complex not really belonging to the lecithin molecule; if so, it must have the same general properties as the lecithin itself, both in regard to solubility in ether and precipitation by acetone, and is likely present in many so-called lecithins. Of course it represents only a comparatively small part of the total lecithin nitrogen.

In the hydrolysis experiments carried out with lecithin-cadmium-chloride mentioned above, it was found that the residue obtained gave, as usual, a distinct indication of the presence of nitrogen; as before it was found quite impossible to get rid of this however prolonged washing was attempted; as in lecithin, it seems certain that this residual insoluble nitrogen has nothing to do with choline.

Since it has been shown that on the hydrolysis of lecithin a considerable percentage of nitrogen actually present in the end alcoholic solution is not precipitated as choline-platinum chloride, this nitrogen ought to be present in the filtrate after the separation of the platinum chloride precipitate. That the substance obtained was really the double platinum salt of choline is indicated by the results of ignition experiments, which generally gave a residue of platinum but slightly wide of the theoretical amount: it is, of course, quite likely that in some cases,

at any rate, the salt was not of *absolute* purity, but any admixture of other material must have been so slight as to be of little or no importance in judging of the general results. To substantiate this some experiments were made in order to ascertain if the amount of nitrogen used up actually corresponded to the amount necessary for the quantity of choline-platinum-chloride isolated. This was found to be so, the remainder of the nitrogen being present in the filtrate.

A number of these final filtrates were now united, some water added, and the excess of platinum separated by means of H_2S, the solution being heated during the passage of the gas. It was then filtered from platinum sulphide and the clear filtrate evaporated to dryness: residue was then dissolved in a small quantity of absolute alcohol and again treated with platinum chloride: in each case a slight precipitate was obtained, but it invariably proved to consist of a mixture of a small amount of choline-platinum-chloride with some of the barium salt of platinum chloride. This was in turn filtered off and filtrate again treated with H_2S, as above. The final solution obtained was then treated with various general precipitating reagents, but no definite substance could be isolated in this way. Experiments in this direction are at present being carried out, but it seems certain that the excess nitrogen of lecithin is not present as an ordinary basic compound; on the other hand there is some evidence that part of it, at any rate, is present in the form of an amino-acid, but this will be entered into in a later communication. The purpose of the present investigation was to ascertain the reason why in the hydrolysis of lecithin the actual amount of choline isolated should invariably fall so far short of the theoretical amount, and also to obtain some definite evidence with regard to the correctness or otherwise of the generally accepted formula with regard to its nitrogen distribution.

In the light of the above experiments it seems to me that the reason for the discrepancies mentioned depend on the fact that different lecithins contain varying amounts of choline; also that the ordinary formula cannot be accepted. Again, it is likely that many of the specimens formerly examined contained mixtures of other phosphatides besides lecithin, and so gave even lower results than recorded above.

With regard to other phosphatides examined mention may be made of two—the mon-amino-diphosphatide isolated from the heart muscle by Erlandsen, and from eggs by the writer. As mentioned in a former paper these two substances differ somewhat in their general analyses, but in each case no definite evidence of choline or any other substance of basic nature could be obtained. On hydrolysis in the usual way, platinum

chloride may give a slight often ill-defined precipitate, but the amount is so exceedingly small even when a large quantity of the substance is used, as to make it impossible to say what it really consists of. It would seem to be more of the nature of an impurity, and is in no way equivalent to the amount of nitrogen present. With the comparatively small amounts of substance at my disposal it is difficult to make any definite statement, but so far, in the case of the egg phosphatide, I have failed to obtain any substance of a basic nature in the ordinary sense though many experiments in this direction have been made. At present a large supply of heart 'cuorin' has been prepared, and it is hoped that some future experiments with large amounts of substance may settle the point.

The general results of the above investigation and the chief conclusions inferred in the light of these results may be shortly summed up as follows:—

Summary

From different lecithins (heart, egg, etc.) prepared with the greatest care, and having a ratio of N : P as almost 1 : 1, different amounts of choline are obtained under similar conditions. On the other hand the amount obtained from any given specimen is practically constant, the result being the same whether watery or alcoholic solution of $Ba(OH)_2$, or watery solution of hydrochloric acid be employed. From this it is obvious that these different lecithins, though showing somewhat similar figures as the result of elementary analyses, are not identical in composition, particularly with regard to their nitrogen distribution.

A long series of experiments with choline chloride has shown that if choline is really present at the beginning of hydrolysis, a very large percentage of it can be ultimately recovered as the double platinum salt, and the presence of substances corresponding to the known decomposition products of lecithin do not, by means of any more or less obscure reaction with the choline present, prevent this base being ultimately recovered as the double platinum salt; in one experiment containing excessive amounts of all the known products of hydrolytic decomposition of lecithin, there was a loss of only 5 per cent. of the choline, obviously due in great part to the difficulty experienced in thoroughly washing the bulky residues present.

Since these insignificant losses entirely fail to account for the small yield of choline obtained from lecithin, it is necessary to assume that the whole of the nitrogen of lecithin is not represented by choline.

In some lecithins, however, more of the nitrogen present is actually

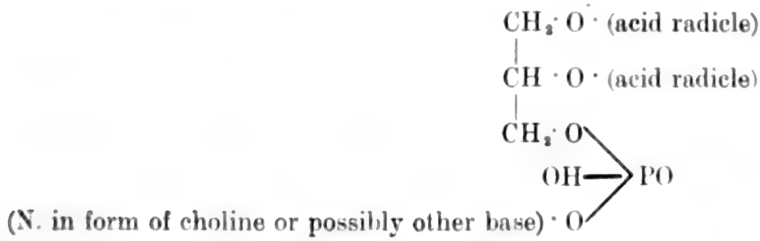
represented by choline than is the case in other lecithins, a comparison of the amount of choline-platinum-chloride salt obtained from heart and egg lecithin, respectively being roughly 42 per cent. and 65 per cent, while from a trade lecithin 80 per cent. was obtained.

Results substantiating the above were obtained on precipitating egg lecithin with cadmium chloride and examining the filtrate and salt; in the sample examined some nitrogen was split off by $CdCl_2$ in a form soluble in H_2O; this nitrogen did not represent choline. The cadmium-lecithin-chloride salt contained 10 per cent. more choline (based on its N-content) than the lecithin from which it was obtained. This practically proves that some of the nitrogen originally present in the lecithin was not present as choline.

Other evidence with regard to direct estimation of nitrogen and choline in different filtrates all points in the same direction, and lends additional weight to the inferences derived from a consideration of the above experiments. Whether lecithin is really a chemical unit, or a mixture of two or more substances having the same properties does not affect these facts.

CONCLUSIONS

1. In the light of the results obtained, it is the opinion of the writer that the generally accepted lecithin formula, i.e.—



in which the whole of the nitrogen present is represented by a base—choline *is incorrect, and can no longer be accepted*; probably part of this nitrogen at any rate is present in the form of amino-acid.

2. Lecithins obtained from different sources differ, often to a great extent, with regard to the percentage of nitrogen actually represented by choline, and though often giving somewhat similar results on elementary analysis, cannot be regarded as identical chemical substances.

3. These results fully explain the general failure of former investigators to obtain anything corresponding to the theoretical yield of choline from lecithin.

A POLARIMETRIC STUDY OF THE SUCROCLASTIC ENZYMES IN *BETA VULGARIS*

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Notwithstanding the large number of exhaustive investigations which have been recently carried out on the sugar beet the actual mechanism of the sugar synthesis seems to be practically unknown. Two alternatives seem possible, the simpler of which is that both glucose and fructose are formed in the leaf region and are there subsequently transformed into sucrose which passes by means of the leaf meristem directly to the root. The alternative explanation, that the disaccharide is a degradation product of starch, seems unlikely. Little or no starch is found under normal conditions in the growing leaf and, moreover, such a change would involve the partial transformation of maltose or glucose into fructose. This would necessitate a fundamental change in the sugar molecule, which seems improbable. Again, according to Grafe, no definite sugars have ever been isolated in the degradation products of cellulose, and thus the latter does not seem to be a likely source of the sucrose.

The first alternative is supported by many arguments. Friedrick Strohmer in his valuable contribution to the Wiesner Fest-Schrift[1] shows conclusively from the result of his own experiments and those of A. Girard that the formation of sugars is restricted to the leaf region and, moreover, that the roots of adult plants contain no reducing sugars save in the early stages of growth.

It must be maintained that the evidence seems to be increasing that formaldehyde is one of the initial substances formed during the natural process of photo-synthesis of sugars, and it would thus appear that a close parallel can, in this case, be drawn between natural and artificial

1. *Über Ausspeicherung und Wanderung des Rohrzuckers in der Zucker-Rübe.*

synthesis. Fenton's recent successful reduction of carbon-dioxide to formaldehyde[1] and the earlier work of Usher and Priestley,[2] who showed that the same reaction proceeded in plant cells, has strengthened the argument considerably. There seems little doubt that the artificial sugars obtained from formaldehyde contain large quantities of ketoses, and thus is can be readily understood that in the leaves of the beet the necessary constituents for the formation of cane sugar may be formed. The final stage of the process, the condensation of the glucose and fructose, although theoretically a simple reaction, is one which the organic chemist has been unable to duplicate.

Perhaps the most notable example of the comparative failure of ordinary chemical methods to produce naturally-occuring compounds is to be found in the meagre results which have attended attempts to synthesis disaccharides in the laboratory. Moreover the methods followed in the few successful cases must of necessity be widely different in their nature from those of the natural process. In view of these facts Grabe's statement that the fundamental rôle in the carrying out of these reactions must be ascribed to enzymes seems justifiable.[3]

Assuming that cane-sugar is formed by enzyme action, two alternative theories may be offered. The reaction may be occasioned either by a special enzyme, termed the associating enzyme, capable of condensing glucose and fructose, or the hydrolytic action of invertase may be a reversible change which is capable under suitable conditions of producing sucrose from the constituent sugars. This latter view is upheld by Gonnermann and Stoklasa, and brings the reaction into line with the cases of reversible zymolysis studied by Croft Hill. This idea has also recently received considerable support in the results obtained by Kohl,[4] who subjected invert sugar solutions to the action of yeast extract rich in invertase. A series of titrimetric estimations showed that an equilibrium point was reached, presumably owing to the partial formation of sucrose. The result is interesting, but is not altogether in agreement with the nature of reversible change, as apparently conditions were realised in which both the fructose and glucose contained in the invert sugar completely disappeared on continued action. Pantanelli,[5] in a paper on a similar topic, contributes the remarkable statement that

1. *Trans. Chem. Soc.*, Vol. XCVIII, p. 687, 1907.
2. *Proc. Royal Soc.*, Vol. LXXVIII, B, p. 318, 1906.
3. Macchiati, *Comp. Rend.*, Vol. CXXXV, p. 1128.
4. *Beih. Bot. Centralblatt*, Vol. XXIII, 1, p. 64b-64o, 1908.
5. *Rendiconti Accademia Lincei*, 5, Vol. XVI, pp. 419-428, 1907.

concentrated solutions of invert sugar undergo partial reversion when preserved at room temperature, 'particularly when the solution is feebly alkaline.' The reaction was accelerated by the addition of fungus revertase. The first conclusion seems to have been arrived at by the observation that the reducing power of the solution diminished. In view of Lobry de Bruyn and van Ekenstein's work[1] on the interconversion of hexoses in the presence of alkalies the result seems capable of a simpler interpretation.

Although opinion, in the meantime, seems undivided in attributing sucrose formation in plants to enzyme action, it would appear that it is still doubtful if the action is due to a definite specialised enzyme or is merely the result of reversible zymolytic change.

The following research was undertaken in the hope that the biochemical formation of sucrose by enzyme action could be detected polarimetrically, and for evident reasons the sugar beet was selected for experiment. The adoption of the polarimetric method seemed in this case specially advisable as affording a more accurate index of alteration in the composition of sugar mixtures than any method based on the quantitative use of Fehling's solution.

In view of the fact that the chemical activity of many enzymes is increased by the presence of other enzymes, no attempt was made to separate, even approximately, the individual enzymes from the various mixtures obtained. The total product was tested in its action towards suitable optically active substrata, and in view of the well-defined selective action of enzymes, the method seems justifiable. As far as possible, substrata were used which would readily react and would give large polarimetric differences. The optical activities were determined with an instrument displaying a tripartite field and sensitive to $\frac{1}{100}$ of a degree. Throughout the work standard two-decimetre tubes were used; these were provided with a water-jacket and all the determinations were made at 20° C., accurate to $\frac{1}{20}$°. As a rule the sodium-flame was used, except in special cases where, on account of the unavoidable turbidity of the solutions, the incandescent light was substituted.

Some difficulty was occasionally experienced in filtering the solutions successfully, but this was overcome by shaking with finely-divided ignited silica, a method which we found did not introduce any experimental error.

For the purposes of the present investigation only the examination of the enzymes capable of reacting with carbohydrates and glucosides

1. *Rec. trav. chim. Pays-Bas*, Vol. XIX, p. 1, 1900.

was necessary, and accordingly in the first place the general nature of the soluble enzymes present in the leaves of the adult plant was studied. The plants used weighed on an average about 650 grams. The leaves were detached at the junction of the stem, and after being finely divided, were macerated with water in a sterilised mortar. The pulp thus obtained was mixed with a large excess of water (two litres), and kept in a thermostat at 30° C. for five hours, during which time the mass was kept thoroughly mixed by means of a powerful mechanical stirrer. The liquid was then filtered under pressure and the bright-red filtrate rapidly diluted with four times the volume of alcohol. The mixed enzymes were thus obtained in the form of a grey precipitate, which was filtered off, washed with aqueous alcohol and dried in a vacuum. The dry product gave very little inorganic residue on ignition, and was devoid of any action on Fehling's solution even on prolonged boiling. The solubility in water was slight, one gram of the substance requiring about 700 c.c. of water to effect complete solution at 25° C. The aqueous solution, which became turbid on the addition of traces of alkali, showed practically no activity when examined in a two-decimetre tube. This result was unexpected, but seems to be due to compensation, as enzymes of opposite activity were afterwards shown to be present.

In testing for the presence of probable enzymes, standard solutions of the active substrata were mixed with excess of the solid enzymes, and the mixture sterilised by the addition of a few drops of toluene. The initial specific rotation was determined without delay, after which the liquid was kept in a thermostat at 30° C., polarimetric readings being taken every twelve hours. In each case a control experiment, in which no enzyme was used, was conducted under parallel conditions, and results were only accepted as positive when these control solutions showed no alteration in rotatory power. The following table shows the principal results obtained:

	Enzyme	Substrate	$(\alpha)_D^{20°}$ initial	$(\alpha)_D^{20°}$ final	Diff.
1.	Invertase	...Sucrose ...	+ 66·6° ...	+ 64·4° ...	2·2°
*2.	Emulsin	...Amygdalin ...	— 34·4° ...	— 34·4° ...	nil
3.	Diastase	...Starch ...	— ...	+ 52·0° ...	
4.	Maltase	...α-methyl-glucoside	+ 157·5° ...	+ 128·4° ...	29·1°
5.	Lactase	...Lactose ...	+ 53·1° ...	53·4° ...	0·3°

*In view of the possibility that the enzyme action might be inhibited by the hydrocyanic acid produced from amygdalin, other substrata were afterwards used but a negative result was also obtained.

In the case of Experiment 3, the dilute starch paste used was rapidly liquefied and gave a dextro-rotatory filtrate. The concentration of

dissolved matter was estimated by evaporation of an aliquot part of the solution and weighing the residue dried at 100° C. In this way it was shown that the specific rotation of the solution gradually decreased from + 108°, when the first reading was made, to the constant value + 52°. This ultimate complete conversion into glucose through the intermediate formation of a more highly rotatory compound is in agreement with the idea put forward by Payn[1] that the hydrolysis of starch in the joint presence of diastase and maltase passes through the following stages:—

Starch → Dextrine → Maltose → Glucose

The chemical activity of the leaf enzymes used in the above experiments was somewhat disappointing, few positive results being obtained. This seems to be due to the method of preparation and purification which would yield only the more soluble enzymes. A similar result has already been obtained by Brown and Morris[2] in a study of the enzymes present in foliage leaves. It seems in fact to be a general experience that careful filtration of the original aqueous extract is not desirable in the preparation of enzymes as the product obtained displays very little reactive power. Better results are, as a rule, given by straining the liquid extract of the macerated tissues through fine muslin, and a similar method was therefore adopted in the case of the leaves of the adult beet.

Preparation of Mixed Enzymes from the Leaf

The leaves were macerated with water and extracted in the thermostat as already described, the resulting liquid being strained through several folds of fine muslin. On adding a large excess of alcohol to the turbid filtrate, a brown sludge separated from which the supernatant liquid was decanted away. After washing well with absolute alcohol, the insoluble matter was filtered under pressure and washed with 50 per cent. alcohol until the washings were inactive and ceased to reduce Fehling's solution. The purified precipitate was then diluted with water so as to give a mixture containing 1 per cent. of the dry enzymes, and the liquid was rendered antiseptic by the addition of a little toluene. A filtered sample was optically inactive and did not reduce Fehling's solution.

This method of obtaining the enzymes suspended in water neces-

1. *Comp. Rend.*, Vol. LIII, p. 127.
2. *Trans. Chem. Soc.*, Vol. LXIII, p. 604, 1893.

sitates a modification in the preparation of the test-solution. Where possible, 20 per cent. aqueous solutions of the substrata were prepared, and 25 c.c. introduced by means of a pipette into a standard 50 c.c. flask, which was then diluted to the mark with the homogeneous enzyme sludge. The control solutions were similarly prepared, the dilution to half the original concentration being of course made with water. The experimental solutions were filtered through several baryta filters and the optical rotations determined without delay. All the solutions were kept in the thermostat at 35° C., the rotations being determined at regular intervals. The following positive results were obtained:

Substrate used	c	$(\alpha)_D^{20°}$ initial	$(\alpha)_D^{20°}$ final	difference
α-methylglucoside ...	9·995 ...	+ 157·0° ...	+ 130·6° ...	27·4°
Sucrose ...	10·006 ...	+ 66·2° ...	+ 31·4° ...	34·8°
Inulin ...	1·2502 ...	— 34·2° ...	— 38·4° ...	4·2°
Starch ...	— ...	— ...	+ 60·0° ...	—

The results confirmed those already obtained with the purified enzymes, but the actions were much more rapid. Although marked optical differences were observed in a few hours the experiments were in each case continued for twenty days. The joint presence of maltase and diastase was confirmed, while inulase and lactase were again shown to be absent. In this case, however, evidence was obtained that invertase was present, as shown by the following figures:—

Experiment—Concentration of sucrose	= 10·0060,	initial $(\alpha)_D^{20°}$	= + 66·2°
		final $(\alpha)_D^{20°}$	= + 31·4°
Control— ,, ,,	= 10·0060,	initial $(\alpha)_D^{20°}$	= + 66·6°
		final $(\alpha)_D^{20°}$	= + 63·2°

The result was verified in a duplicate experiment, but in no case was complete inversion obtained.

Examination of the Enzymes from the Stem and Root Regions

After removal of the leaves, the roots were separated from the short stem region and the enzymes isolated as described above in the form of an aqueous sludge. Prolonged washing with dilute alcohol was, of course, necessary to completely remove sugars and also the red colouring matters. In addition, the bulky solid residue left in each case on the muslin filter after extracting the macerated organs with water was utilised in the following manner: The material, after being washed with water, was spread on sheets of filter paper and dried in a current of

air at 30° C. After several days' treatment, the brittle residue was powdered finely in an agate mortar, again digested with a large excess of water, filtered and dried. In this way we were able to avoid the extensive decomposition which results when attempts are made to desiccate the tissues without in the first place removing all soluble organic matter. A similar method has been used by Brown and Morris in demonstrating the presence of diastase in foliage leaves[1]. We thus obtained four preparations, viz.:—stem-sludge, solid stem, root-sludge, and solid root, all of which were found to be capable of promoting hydrolytic changes. The average yields obtained were:—

Weight of stem used ...	133 grams.	Weight of solid stem obtained ...	6·5 grams.
Weight of root used ...	520 grams.	Weight of solid root obtained ...	19 grams.

As usual each preparation was found to be inactive and to be devoid of any action upon Fehling's solution. In preparing the test solutions the method already described was adopted, that is, an aliquot part of the solution was diluted to half the concentration with the stem or root sludge. In the case of experiments with the solid stem or solid root, half a gram of the powdered tissues was added to 25 c.c. of the test solution, which was then diluted to 50 c.c. with water. The control solutions were subjected to a similar dilution without the addition of any enzymes. Sterilisation was effected by the addition of a little toluene or chloroform, and heating was conducted in the thermostat at 30° to 35° C. The optical changes were complete in a few hours, but the values given below were those obtained after seven days' treatment.

Test for Invertase

Substrate = sucrose ('Kahlbaum'). c (initial) = 5·000 c (final) = 2·500

Stem sludge ...	initial $(\alpha)_D^{20°} = +66·10°$	...	final $(\alpha)_D^{20°} = -12·8°$
Solid stem ...	„ $(\alpha)_D^{20°} = +66·80°$	...	„ $(\alpha)_D^{20°} = +36·0°$
Root sludge ...	„ $(\alpha)_D^{20°} = +66·10°$	...	„ $(\alpha)_D^{20°} = +61·8°$
Solid root ...	„ $(\alpha)_D^{20°} = +66·10°$	...	„ $(\alpha)_D^{20°} = +62·0°$

A well marked difference therefore exists between the results obtained with the root and stem preparations. In the former the optical changes observed were small and did not differ in any marked degree from those shown by the control solutions.

It would thus appear that while invertase is present in the stem region it is absent in the root. The result agrees with the general

1. *Loc. cit.*

observation that this particular enzyme is, as a rule, absent in storage organs during the period in which reserve material is being laid down. Our observation is, however, at variance with those obtained by Gonnermann and Stoklasa.[1]

Test for Emulsin

Substrate = β-methylglucoside c (initial) = 4·060 c (final) = 2·030

Stem sludge	...	initial $(a)_D^{20°}$ = — 33·5°	...	final $(a)_D^{20°}$ = — 22·5°
Solid stem	...	„ $(a)_D^{20°}$ = — 33·5°	...	„ $(a)_D^{20°}$ = — 22·1°
Root sludge	...	„ $(a)_D^{20°}$ = — 33·5°	...	„ $(a)_D^{20°}$ = — 22·7°
Solid root	...	„ $(a)_D^{20°}$ = — 33·5°	...	„ $(a)_D^{20°}$ = — 25·1°

The above results were obtained twenty-four hours after commencing the experiments; meanwhile the control solutions suffered no alteration in rotatory power. As in each case the liquids reduced Fehling's solution actively after treatment with the enzymes, the results are accepted as indicating the presence of emulsin in all the preparations. As a confirmatory test, salicin was substituted for β-methylglucoside as a substrate. In each case the result was the same, the specific rotations of the solutions diminishing about 14° in twenty-four hours, and the presence of both glucose and saligenin was detected in the resulting liquids.

Test for Maltase

Maltase was found to be present in both the root preparations, but was absent in the stem as shown by the following typical results:

Substrate = a-methylglucoside c (initial) = 4·9992 c (final) = 2·5005

Solid root	...	initial $\left[a\right]_D^{20°}$ + 156·0°	final $\left[a\right]_D^{20°}$ + 140·0°	diff. =	16·0°
Solid stem	...	„ + 156·0°	„ + 155·4°	„	0·6°

Substrate = Maltose (Kahlbaum) c (initial) = 5·0010 c (final) = 2·5005

Solid root	...	initial $\left[a\right]_D^{20°}$ + 132·1°	final $\left[a\right]_D^{20°}$ + 96·8°	diff. –	35·3°
Solid stem	...	„ + 132·1°	„ + 128·4°	„ =	3·7°

In using maltose as a substrate we took the precaution of adding a minute trace of caustic soda to the solution in order to promote mutarotation. The optical changes observed are therefore not due to stereöchemical alterations in the sugar.

1. Czapek's *Biochemie der Pflanzen*, Bd. I, p. 375.

Test for Diastase

Substrate Starch solution c (initial) approx. 0·50

Stem sludge ...	no initial a taken	...	final $[a]_D^{20°}$	+ 44·0°
Solid stem ...	,, ,,	...	,,	+ 32·0°
Root sludge ...	,, ,,	...	,,	+ 24·0°
Solid root ...	,, ,,	...	,,	+ 28·0°

The final concentrations were determined by evaporation of a measured volume of the solutions and weighing the residue dried at 100° C. All the products reduced Fehling's solution actively even in the cold. We are in the meantime unable to explain the fact that the end values obtained are lower than those calculated for glucose; no β-glucose was present as the rotations remained permanent on adding a trace of alkali. The concentration would of course only be approximate, but any experimental error thus introduced would be insufficient to account for the discrepancy between the calculated value for glucose and those experimentally found. Possibly inactive products may have been formed, or a partial conversion into other hexoses may have taken place. It will be noticed that in the case of enzymes extracted from the leaf the hydrolysis of starch resulted in the formation of a reducing sugar which displayed the correct rotatory power for glucose.

Summary of the Distribution of Sucroclastic Enzymes in the Adult Beet

(1). *Invertase.* Invertase was not detected in the carefully purified mixture of enzymes obtained from the leaf. When, however, the enzymes are obtained by filtering through muslin and are afterwards washed free from reducing sugars, invertase is then found to be present.

Both the aqueous sludge containing the enzymes of the stem and also the powdered stem contain invertase.

Similar preparations from the root contain no invertase.

(2). *Diastase* was found in all the preparations examined.

(3). *Maltase* is present in the leaf and root regions, but was not detected in the stem preparations.

(4). *Inulase* seems entirely absent in the leaf region. Positive results were obtained with all the preparations from the stem and root, but the optical changes observed were irregular.

(5). *Emulsin.*--The enzymes obtained from the leaf gave negative results using amygdalin as a substrate; the stem and root preparations on the other hand gave positive results with salicin and β-methyl-glucoside.

(6). *Lactase* was absent in every case.

Attempted Reversible Zymolysis by the Action of Beet Enzymes

The methods adopted in the attempted condensation of glucose and fructose by enzyme action were similar to those successfully applied by Croft-Hill[1] in the enzymatic synthesis of maltose from glucose. As sucrose is incapable of forming an osazone, and as no suitable insoluble derivative is known, the separation of which would serve to detect the formation of the sugar in the presence of monosaccharides, recourse was again had to a polarimetric method of following the reaction. The plan adopted was to subject concentrated sterilised solutions of invert sugar to the action of the various enzyme preparations, polarimetric readings being taken at intervals. Change in rotation cannot, of course, in itself be considered evidence of sucrose formation. Other factors may play a part, including the possible self-condensation of both glucose and fructose. The experiments were therefore extended by hydrolysing the products and ascertaining if the optical value for invert sugar was reproduced. This joint evidence of a fall in laevo-rotatory power due to enzyme action, and a subsequent increase to nearly the initial value of hydrolysis, would justify the conclusion that condensation of the hexoses had taken place. In the absence of positive results indicating self-condensation of glucose or of fructose this double change in rotation would be significant of the presence of sucrose, or at all events, of a glucosidic compound containing both glucose and fructose. Before commencing our observations several preliminary experiments were necessary. The preparation of a suitable solution of invert sugar, displaying the maximum rotatory power, presented some difficulty. In the first place pure crystallised glucose and fructose ('Kahlbaum') were dried until constant in weight, the former at 110° C., and the latter in a vacuum. Equal weights of each sugar, accurate to 1 milligram were then dissolved in water in a standard flask and made up to the mark at 20° C. After allowing ample time for mutarotation to take place, the rotations were determined in a two-decimetre tube ($t = 20°C.$). The result was, however, unsatisfactory, as the values obtained were not very uniform and were invariably too low. This is shown in the following table:—

1.	c. for glucose = 20·0010	c. for fructose = 20·0013	$(a)_D^{20°}$	= − 21·2°
2.	c. for glucose = 20·0015	c. for fructose = 20·0005	$(a)_D^{20°}$	= − 19·1°
3.	c. for glucose = 20·0006	c. for fructose = 20·0003	$(a)_D^{20°}$	= − 19·8°

1. *Loc. cit.*

As the correct value for invert sugar is $(a)_D = -24{\cdot}7^\circ$ it would appear that this method of directly weighing out the sugars is inapplicable, probably owing to the difficulty in obtaining fructose in an anhydrous condition. Although the solutions referred to above were used in preliminary experiments, we prepared our invert sugar solutions by the method recommended by Maumené.[1] Pure sucrose (Kahlbaum) in quantities of 150 grams was dissolved in water (250 c.c.), and heated in sealed flasks at 106° C. for fifty hours. The resulting liquid gave $(a)_D^{20^\circ} = -24{\cdot}1^\circ$ calculating on the complete conversion into glucose and fructose. The effect of hydrolytic agents on this solution was then studied in order to ascertain if any sucrose remained unaltered. A test portion was diluted to half the concentration with water containing varying amounts of hydrochloric acid, and the optical changes on heating observed. It is of course well known that hydrochloric acid, even in dilute solution, readily decomposes fructose, and consequently suitable conditions of acid, concentration, and temperature had to be determined which would suffice to hydrolyse sucrose and yet be without action on fructose. Using solutions containing 5·3, 2·6, and 1·3 per cent. of acid the laevo-rotation of the invert sugar diminished steadily when the liquid was maintained at 50° C. At 20° C., however, although a similar fall in laevo-rotation was recorded in the case of the strongest acid solution, a constant value was ultimately obtained. On the other hand, the solution containing 2·6 per cent. of acid remained unaltered in rotatory power when kept for six days at 20° C. This result proved the complete absence of sucrose, and gave the desired conditions for carrying out a polarimetric test for sucrose formation in the subsequent experiments.

In the first place the precipitated enzymes obtained from adult leaves were used, but, as was expected from the feeble activity of the preparation, no positive results were obtained. Even after an interval of two months the optical activity of an invert sugar solution was found to be quite unaltered by the action of the enzymes. Recourse was therefore had to the more active enzyme preparations previously referred to as the 'stem sludge' and the 'root sludge.' Only small optical changes were, however, observed, but these were in the right direction, and were supported by the evidence of hydrolysis. The results of one typical experiment may be quoted:—

Initial concentration of invert sugar solution	=	40·0100
Concentration when diluted with stem sludge	=	20·0050
Initial specific rotation of the mixture	=	— 24·7°
After 350 hours' action at 35°	=	— 21·2°
Decrease in laevo-rotation due to enzyme action	=	3·5°
Increase in laevo-rotation due to subsequent hydrolysis	=	2·1

1. *Journal des fabricants de sucre*, Vol. XXXI, p. 46.

Control experiments showed that the enzymes were without action on either glucose or fructose solution alone. It would thus appear that the glycolytic enzymes described by Stoklasa are without action on concentrated glucose solutions. The above data represents about the average result obtained in five different experiments, and corresponds with the formation of approximately 4 per cent. of sucrose. The addition of maltose did not effect any appreciable improvement in the process and, moreover, prevented the hydrolysis being studied.

It must be admitted, even if the above results can be correctly ascribed to sucrose formation, that the magnitude of the changes are small. It appeared possible that this might be due to alteration in the nature of the associating enzyme once the storage of sucrose had ceased, and, consequently, in another series of experiments, young plants, which had not begun storing, were used as the source of the enzyme preparations. The isolation of the mixed enzymes was carried out as already described, but no differentiation into leaf and root region was made, the entire plants being macerated and extracted. The sludge finally obtained consisted of a mixture of 445 grams of the mixed enzymes diluted to a litre with water.

The following experiment showed that the sludge possessed associating powers:—

70 c.c. of invert sugar solution giving $(a)_{D}^{20^{\circ}} = -23{\cdot}7^{\circ}$ were introduced into a standard 100 c.c. flask and made up the mark with the homogeneous sludge. A portion filtered at once gave in a one-decimetre tube the rotation $a_{20} = -6{\cdot}86^{\circ}$. The solution was then kept at 35° C. in a thermostat for a week, when it was found that the rotation had diminished to $-5{\cdot}95^{\circ}$. The subsequent optical changes were only small, and the constant value observed was $a = -5{\cdot}70^{\circ}$. The total change in a therefore amounted to $+1{\cdot}16^{\circ}$. Expressed in specific rotations the change becomes $(a)_{D}^{20^{\circ}} = -23{\cdot}9^{\circ} \rightarrow (a)_{D}^{20^{\circ}} = -19{\cdot}8^{\circ}$, and is, moreover, in the right direction. The subsequent hydrolysis was carried out by diluting a test portion to half the original concentration with water containing sufficient hydrochloric acid to give a 2·6 per cent. acid solution. The initial rotation was $(a)_{D}^{20^{\circ}} = -19{\cdot}3^{\circ}$, and on standing at 20° this gradually altered to the constant value $(a)_{D}^{20^{\circ}} = -22{\cdot}7^{\circ}$, the actual change in a being $-0{\cdot}99^{\circ}$. This result can only be explained on the assumption that a glucosidic product had been formed possessing a less laevo- or more strongly dextro-rotation than invert sugar, and which is, moreover, capable of undergoing hydrolysis to give an equimolecular mixture of glucose and fructose. Assuming

that sucrose is the only glucosidic product thus formed, the result indicates the production of about 6 per cent. of the disaccharide. The above observation was controlled by a duplicate experiment in which a sample of the invert sugar solution was diluted with water in place of the enzyme sludge and kept in the thermostat for an equal time. No appreciable alteration in rotatory power was, however, observed even after three months. It was also shown that the sludge was without action on glucose or fructose solutions alone; only in the joint presence of both sugars were any optical alterations observable.

These results are summarised below:—

Nature of Experiment	Length of tube in decimetres	Initial $\alpha_{20°}$	Final $\alpha_{20°}$	Δ	Behaviour on hydrolysis
Invert sugar and enzyme sludge	1	— 6·86	— 5·75	16·6	— 5·55° $\rightarrow$ — 6·54°
Control sol. of invert sugar	1	—14·43	—14·37	0·4	No change
Glucose (20 %) and enzyme sludge	2	20·21	20·13	0·4	No change
Fructose (20 %) and enzyme sludge	2	—34·65	—34·80	0·4	No change

The column marked Δ contains the optical changes expressed in percentages, and it will thus be seen that a marked difference exists between the first experiment and the subsequent controls.

The result was verified in a duplicate series of experiments in which the treatment with the enzymes was continued for three months: once more the control solutions gave perfectly negative results, but the invert sugar solution underwent similar alterations to those quoted above:—

Initial $(\alpha)_D^{20°} = -24·05°$ Final $(\alpha)_D^{20°} = -18·10°$. Diff. $= +5·95°$.

Change of rotation on hydrolysis $(\alpha)_D^{20°} = -18·1° \rightarrow -27·2°$.

A slight irregularity will be noticed in the permanent end value obtained on hydrolysis. This we are in the meantime unable to explain, but nevertheless it is evident that the rotation changes are of the same nature as those discussed above, and are in agreement with the idea that about 6·6 per cent. of sucrose had been produced in the reaction.

It is of course premature to claim the above results as a successful bio-chemical synthesis of sucrose, but it is difficult to find any other explanation of the optical changes other than that already suggested, viz., that the aldose and ketose combine to form a glucosidic derivative, and sucrose is the only compound of this type now known.

Discussion of the Results

The considerations of interest to the plant physiologist accruing from this work group themselves around the following heads:

(*a*) The topography and work of the enzymes.

(*b*) The synthesis of a glucosidic compound, presumably sucrose, by the action of beet sludge on invert sugar.

(*c*) The difference in magnitude of the results obtained *in vivo* as contrasted with that *in vitro*.

(*a*) 1. The topography of the enzymes here worked out may be tabulated thus:—

	Leaf	Stem	Root
Invertase	+	+	0
Diastase	+	+	+
Maltase	+	0	+
Inulase	0	+	+
Emulsin	0	+	+

A significant feature in this enzyme distribution is the absence of invertase from the beet root. Kastle and Clark[1] found that in inulin- and starch-producing plants such as the artichoke and the potato, even in the tubers, when the inulin and starch were undergoing storage, invertase was present in larger quantity than either inulase or diastase. In the beet root, when sucrose is being stored the appropriate enzyme for its formation and hydrolysis is absent. Since in the artichoke and potato respectively the inulin and starch are formed *in loco*, the conclusion to be drawn, on analogy, in regard to the beet seems to favour the hypothesis that sucrose is not formed *in loco* in the root, but is only stored there after undergoing translocation as sucrose from the other organs of the plant.

(*a*) 2. The varied associations of the enzymes in the different plant organs is a striking point. To take the case of diastase, which occurs in all parts of the beet, it is found that in the leaf it is associated with invertase and maltase, in the stem with emulsin in addition, while in the root a third variation in the environment of activation of diastase is produced by its association with maltase, inulase and emulsin. The questions which naturally suggest themselves are as to whether the diastatic activity is affected by the different environment in each case, and as to whether a given combination of enzymes may not inhibit the appearance of some other enzyme which *a priori* might be expected to be present. This latter bears on the absence of invertase in the root in an

1. *Amer. Chem. Journ.*, Vol. XXX, p. 422, 1903.

environment containing an association of diastase, maltase, inulase and emulsin. That some interaction of the enzymes of an acceleratory, retardatory or inhibitory nature occurs is quite in agreement with the doctrine propounded on the result of experimental research by several investigators[1].

(*a*) 3. Besides the differences in the environmental spheres of activation resulting from the varied association of the enzymes themselves in the different plant organs, other factors enter to complicate the result. For example, the associated enzymes of the leaf are subjected to different light conditions from those in the root; in the leaf to alternation of light and darkness, in the root to continuous darkness. That light affects the results in the case of invertase action is shown from the recent researches of Kohl,[2] who has found that in darkness inversion gives place to the opposite process sooner than in light. Girard's discovery[3] that the amount of sucrose in the beet leaf is much increased towards evening may bear directly on this point. Other factors affecting the result are, the presence of products of hydrolysis, differences in the osmotic pressure in the cells of various organs, and differences in the reactions of the cell contents. In the last two cases the 'intensity factor'[4] would be affected, on the one hand by alteration in the concentration of the enzyme and on the other from the acceleration or retardation of the enzyme action due to increased or diminished acidity.

(*b*) 1. Of the experiments on reversible zymolysis the first series made with pure enzyme extracts of beet leaves on invert-sugar substrates gave no positive results. It is, however, a common experience that pure enzyme extracts are less active than sludges. This was strikingly shown in the negative results obtained in testing for invertase by the action of the leaf extracts on sucrose, whereas, when the sludge was substituted, invertase was proved to be present. The presumption is that by the action of the leaf sludge on invert sugar, reversible zymolysis would have taken place. The second series of experiments with stem sludge on invert sugar gave figures corresponding with the presence of 4 per cent. of sucrose—a result to be expected from the ascertained presence of invertase in the stem. The third series with the sludge of seedling plants gave the higher figure (6 per cent. sucrose), indicating the greater activity of the plant at the beginning of the storage as compared with the diminished activity of

1. Bayliss, *The Nature of Enzyme Action*, p. 657, and references.
2. *Abstr. Bot. Centr.*, Vol. CVIII, p. 137, 1908.
3. *Compt. Rend.*, T. XCVII, p. 1305, 1883.
4. Visser, *Zeit. f. physik. Chem.*, Vol. LII, p. 283, 1905.

the older plant at the end of the storage period. The reasons for regarding the products as sucrose have already been stated.

(*b*) 2. That the cane-sugar stored in the beet root is formed from antecedent monosaccharides by reversible zymolysis in the organs containing invertase (viz., in the leaf and stem), and thence translocated as such seems highly probable.

The alternative view that the sugars travel downwards as monosaccharides and are subsequently condensed into the disaccharide[1] meets the diffusion difficulty. The absence of invertase from the root as here shown, and the results of Strohmer's researches proving that practically no reducing sugars occur in the root, while, according to Girard[2], sucrose is present in all parts of the plant in the earliest stages of development, militate against this view.

(*b*) 3. In relation to the translocatory difficulty in the first view, reference may be made to the researches of Hanstein[3] and Puriewitsch[4] on the diffusion of disaccharides in the maize endosperm, to Peklo's recent discovery[5] that the sieve tubes of the beet serve as the sugar-conducting channels, and to Pfeffer's dictum on the great regulatory faculty of the cell cytoplasm in relation to the materials to be diffused.

(*b*) 4. That the metabolism of the sucrose during the second spring to supply the young shoots takes place in the stem[6] is supported by the fact that invertase is found in that organ.

(*c*) 1. The apparently relatively unlimited quantity of disaccharides capable of being formed in the living plant is in marked contrast to the small results obtained experimentally *in vitro*. This is not surprising when it is remembered that the reaction is presumably a reversible one. *In vitro* the equilibrium stage attained is permanent owing to the fact that the reaction products are not removed, while in the plant the equilibrium phase is only momentary owing to the continual removal of the products as they are formed.

1. Maquenne, *Compt. Rend.*, T. CXXI, p. 834, 1895.
2. *Compt. Rend.*, T. CII, 1887.
3. *Flora*, p. 419, 1894.
4. *Ber. d. Bot. Ges.*, p. 206, 1896.
5. *Bot. Centr.*, Vol. CVIII, p. 239, 1908.
6. Strohmer, *loc. cit.*

THE OUTPUT OF ORGANIC PHOSPHORUS IN URINE

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The existence of organic phosphorus compounds in normal urine has often been asserted and as often denied. In a previous paper (1) I have shown their undoubted existence. The present investigation deals with the quantity of organic P_2O_5 excreted in the urine of healthy persons on an ordinary diet. The results obtained by many previous workers are unreliable owing to the employment of unsuitable methods. Ehrstrom (2), Gumlich (3), Keller (4), Le Clerc and Cook (5), and older workers such as Lépine (6) and Zuelzer (7) attempted to determine organic P_2O_5 by methods entailing titration of urine with uranium acetate. This method gives a value greater than inorganic, indeed sometimes greater than total P_2O_5, so that it is valueless for the determination of organic P_2O_5.

Le Clerc and Cook (5), whose dictum is quoted with approval by F. G. Benedict (8), state that there is insufficient evidence of the existence of organic phosphorus, despite the fact that in twenty-four hours' urine from a dog they obtained a difference of 0·093 gram P_2O_5 between total and inorganic, in rabbits a difference of 0·055 gram. Even though one agree with these workers that the method they employ is not sufficiently exact to afford evidence of the existence of organic phosphorus, one cannot agree that it is sufficiently exact to afford evidence against such existence.

Oertel (9) was the first to use a sound method of estimating organic phosphorus. He precipitated phosphates by means of calcium chloride, and determined organic P_2O_5 in the filtrate. This was evaporated to dryness, fused with KOH and KNO_3, precipitated with ammonium molybdate, dissolved in ammonia, re-precipitated with magnesia mixture, incinerated and estimated as pyrophosphate. The process is thus very lengthy and involves several manipulations.

Oertel obtained values for the output of organic P_2O_5 in twenty-four hours ranging from 0·12 gram (5 per cent. of total P_2O_5) to 0·03 gram, (1·5 per cent. of total P_2O_5). He considers 0·05 gram to be the usual

quantity. Mandel and Oertel (10), employing the same method, found in the twenty-four hours' urine of three individuals an average of 0·024 gram organic P_2O_5, equal to about 2 per cent. of the total.

Bornstein (11) made some observations on the output of inorganic and organic P_2O_5 on an ordinary diet and on plasmon. He estimated organic P_2O_5 in the filtrate after barium chloride, by a method somewhat similar to that of Oertel. His results are here summarised:—

	Nitrogen	Total P_2O_5	Organic P_2O_5	Percentage of P_2O_5 as Organic
Average of 14 days	14·0	2·09	0·058	2·8
Highest values	14·2	1·82	0·16	8·8

Bornstein himself considers that there is some mistake in the highest values, and rejects them in his average.

Bock (12) used methods entailing uranium acetate titration, but also in some cases estimated total P_2O_5 by Neumann's method, and inorganic P_2O_5 with calcium chloride or barium chloride. In rabbits he found as much as 0·29 gram of organic P_2O_5, equal to 11 per cent. of total, in twenty-four hours' urine, in cats from 0·04 gram, 2·5 per cent., up to 0·20 gram, 11 per cent.

The present observations were carried out on the urines of healthy individuals on an every day diet during January, February and March. All these persons followed sedentary occupations. The estimations were carried out by the methods described in a previous paper (1).

Samples of urine from different individuals gave the following values:—

Table I—Inorganic and Organic Phosphorus

Grams P_2O_5 in 100 c.c. urine

	Total P_2O_5	Inorganic P_2O_5	Organic P_2O_5		Percentage of P_2O_5 as Organic
			By difference	In filtrate	
S.	0·092	0·086	0·006	0·004	5·5
K.	0·124 0·121	0·117	0·005	0·005	4·0
P.	0·148 0·145	0·145 0·138	0·003 0·007	-- 0·007	5·0
M.	0·098 0·099	0·090	0·008	0·010	9·0

Since these results were considerably higher than those usually cited, further observations were made on the daily output of different individuals, usually over a period of several consecutive days.

TABLE II OUTPUT OF ORGANIC P_2O_5 IN 24 HOURS

Subject	Day	Quantity	Total Nitrogen	Total P_2O_5 (Grams P_2O_5)	Organic P_2O_5	Percentage P_2O_5 Organic	$\frac{N}{P_2O_5}$ ratio
M. aet. 25	I	1320	—	3·00	0·303	10·1	—
,,	II	1030	—	2·30	0·155	6·7	—
,,	III	1000	—	2·43	0·180	7·5	—
,,	IV	1240	—	2·75	0·148	5·4	—
,,	V	1070	—	2·45	0·225	9·0	—
	Average	—	—	2·58	0·202	7·7	—
M. aet. 25	I	1200	16·44	2·41	0·168	7·0	6·8
,,	II	1140	12·77	1·90	0·114	6·0	6·8
,,	III	1200	15·28	2·45	0·120	4·9	6·2
,,	IV	1050	14·59	2·05	0·121	6·2	7·1
,,	V	1500	15·90	2·05	0·180	7·8	6·9
,,	VI	1050	13·46	2·31	0·140	6·0	6·5
	Average	—	14·74	2·19	0·140	6·3	6·7
L. aet. 27	I	1100	10·18	2·10	0·104	5·0	4·9
,,	II	1230	9·76	1·90	0·041	2·1	5·1
,,	III	1400	11·02	2·41	0·063	2·5	4·6
	Average	—	10·32	2·13	0·068	3·2	4·9
D. aet. 40	I	1850	16·63	2·84	0·27	9·5	5·9
,,	II	1150	14·78	2·61	0·01	0·4	5·6
,,	III	2050	16·43	2·77	0·15	5·5	5·9
	Average	—	15·94	2·59	0·14	5·1	5·8
P. aet. 30	I	1700	13·71	2·32	0·223	10·0	5·9

Organic P_2O_5 averaged 0·15 gram per diem, equal to 6·2 per cent. of total P_2O_5.

The highest output was 0·3 gram, the lowest 0·04 gram.

It will be noticed that the N : P_2O_5 ratio was fairly constant in any one individual, but that it varied greatly in different individuals.

The values obtained for organic phosphorus are considerably higher than those cited by most other workers, even by those who employed accurate methods. This difference must be ascribed in the latter cases to individual variations in output.

THE EFFECT OF INGESTION OF GLYCEROPHOSPHORIC ACID

Although the subject was not on a rigid diet, it was thought worth while to try the effect of adding a large amount of organic phosphorus, in the form of glycerophosphoric acid, to the diet. For several days an approximately similar diet was adhered to, except that on one day glycerophosphoric acid was added. Two series of observations were made on the same subject.

TABLE III—EFFECT OF INGESTION OF GLYCEROPHOSPHORIC ACID

(i) Glycerophosphoric acid (Merck) containing 1·44 grams Organic P_2O_5, 0·075 grams Inorganic P_2O_5 added to diet early on sixth day.

Subject	Day	Nitrogen	Total P_2O_5	Organic P_2O_5	P_2O_5 as Organic	$\frac{N}{P_2O_5}$
M. aet. 25	I-IV (average)	14·99	2·24	0·146	6·4	6·5
,,	V	13·46	2·07	0·140	6·0	6·5
,,	VI*	13·6	2·76	0·165	6·0	4·8
,,	VII	13·77	2·29	0·162	7·0	6·0

(ii) Sodium Glycerophosphate, containing 2·4 grams Organic P_2O_5 and 0·125 grams Inorganic P_2O_5 added to diet on second day.

Subject	Day	Nitrogen	Total P_2O_5	Organic P_2O_5	P_2O_5 as Organic	$\frac{N}{P_2O_5}$
,,	I	14·2	2·47	0·168	6·8	5·7
,,	II*	10·44	3·73	0·113	3·0	2·8
,,	III	10·55	0·16	0·174	8·0	4·9
,,	IV	14·63	2·74	0·150	5·3	5·3

The increase of organic P_2O_5 is well within normal variations—no significance attaches to it. The same might be said of the increase of total P_2O_5, but for the marked alteration in the N : P_2O_5 ratio. It is obvious that a great part of the ingested glycerophosphate has been excreted as inorganic phosphate; it is probable that a considerable portion was not absorbed and would be found in the faeces.

The experiments of Bergmann (13) are of some interest in this connection. He injected into a dog subcutaneously several grams of organic P_2O_5 in the form of glycerophosphoric acid. He found a marked increase in the inorganic P_2O_5, none in the organic. He used titration methods which would only show large changes. The increase in inorganic P_2O_5 was so great, however, as to leave no doubt that the glycerophosphoric acid had been decomposed here without intervention of alimentary processes. It has been asserted that many organic phosphorus compounds are absorbed as such. To test the probability of this assertion, I have subjected sodium glycerophosphate solutions to the action of active preparations of pepsin, of trypsin, and of fresh pancreatic juice, both with and without enterokinase.[1] The solutions were incubated for weeks at 39° C. Inorganic phosphates were estimated at the beginning and at intervals during the experiment. In no case was any increase in the inorganic phosphates found; the glycerophosphate remained unchanged.

1. The juice was obtained from dogs after injection of secretin. It was used without enterokinase because some authors have asserted that enterokinase destroys the lipase present.

It is probable, therefore, that the ingested glycerophosphate in the experiments detailed above was absorbed unchanged. As the glycerophosphate used was synthetic it does not follow that natural glycerophosphoric acid is unaffected by digestive processes.

The Effect of Exercise

This was investigated on two occasions. The urine was collected over four or five days, an approximately regular diet being taken during this period. On the second day a sharp twenty mile walk was taken, on the other days no exercise beyond leisurely walking a couple of miles. The walk was followed on both occasions by slight stiffness, but beyond this no fatigue was felt.

Table IV Effect of Exercise

Subject	Day	Quantity	Nitrogen	Total P_2O_5	Organic P_2O_5	Percentage P_2O_5 as Organic	$\frac{N}{P_2O_5}$
M. aet. 25	I	1440	15·10	2·769	0·181	6·5	5·5
,,	II*	1550	15·23	2·81	0·168	5·9	5·4
,,	III	1200	13·96	2·58	0·114	4·4	5·1
,,	IV	1320	16·01	2·93	0·146	5·0	5·4
,,	V	1510	14·30	2·71	0·110	4·1	5·3
,,	I	1360	13·09	2·18	0·163	7·4	6·0
,,	II*	1710	14·01	2·44	0·171	7·0	5·7
,,	III	1450	15·48	2·70	0·120	4·7	5·7
,,	IV	1250	13·94	2·67	0·134	5·0	5·2

*Twenty mile walk during first half of this day.

These figures do not show any increase of organic P_2O_5 after exercise. The diet was not sufficiently rigid to enable any deductions to be drawn from the nitrogen and total P_2O_5 figures.

No statement can as yet be made as to the origin of the organic phosphorus of urine. As far as can be gathered from the present results and from a long series of observations, on which Dr. Aders Plimmer is at present engaged, the quantity of organic P_2O_5 is not affected by food. It is thought that some indication of its origin may be given by investigation of pathological conditions in which gross changes in lymphoid or nervous tissues are present.

Summary

1. Organic phosphorus compounds are normally present in the urine. Contrary statements are due to the employment of incorrect methods.

2. In young adults on an ordinary diet the organic P_2O_5 was usually more than 0·1 gram per day. Occasionally it fell below this, and in one case it reached 0·3 gram.

3. The percentage of the total P_2O_5 present in organic combination varies considerably from day to day. In the cases examined it averaged 6 per cent. of the total.

4. The addition of a large quantity of organic phosphorus in the form of glycerophosphoric acid to the diet had no distinct effect on the output of organic P_2O_5, while it increased the total P_2O_5 output. Glycerophosphoric acid was not broken down by gastric or pancreatic digestion *in vitro*, so it was probably absorbed unchanged.

5. In the observations made, vigorous exercise was not followed by increased output of organic P_2O_5.

6. The N : P_2O_5 ratio was fairly constant in any one individual on a fairly regular diet. It differed greatly in different individuals, and also in the same individual when the diet was irregular.

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ON THE RELATIVE HAEMOGLOBIN-VALUE OF THE RESISTANT ERYTHROCYTES DURING THE HAEMOLYSIS OF BLOOD WITH HYPOSMOTIC SODIUM CHLORIDE SOLUTION, AND ON THE PERMEABILITY OF THE ERYTHROCYTES TO WATER AS A FACTOR IN THE PRODUCTION OF HAEMOLYSIS

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In a previous paper[1] I have pointed out that the dark coloration described by Wright, and obtained by mixing one part of blood with two parts of a progressive dilution of saline does not represent the point of complete haemolysis. This point is obtained in the observations of McCay[2] and my own observations by mixing one part of blood with two parts of $\frac{N}{40}$ to $\frac{N}{50}$ saline solution. It may, for the sake of convenience, be called *Wright's haemolytic point.*

This point probably represents the stage at which a large number of the erythrocytes undergo haemolysis as the result of osmosis and rupture. The corpuscles that do not haemolyse at Wright's haemolytic point will be termed in this paper the *resistant corpuscles.*

By quantitatively estimating the amount of dissolved haemoglobin in 20 cb.mm. of the supernatant fluid obtained after centrifugalisation of a mixture of blood and two volumes of $\frac{N}{x}$ saline solution, where x is any number from 20 upwards, I have made out the *curve of haemolysis with hyposmotic saline solutions* (see fig. 1).

From the curve below it will be seen that the very beginning of haemolysis starts with $\frac{N}{20}$ saline solution. Then the degree of haemolysis suddenly increases from $\frac{N}{20}$ to $\frac{N}{30}$ saline. From $\frac{N}{30}$ to $\frac{N}{45}$ or $\frac{N}{50}$ it is somewhat gradual, while from $\frac{N}{50}$ upwards it increases very slightly with the higher dilutions.

1. *Bio-Chemical Journal*, Vol. IV, p. 59, 1909.
2. *Ibid.*, Vol. III, p. 97, 1907.

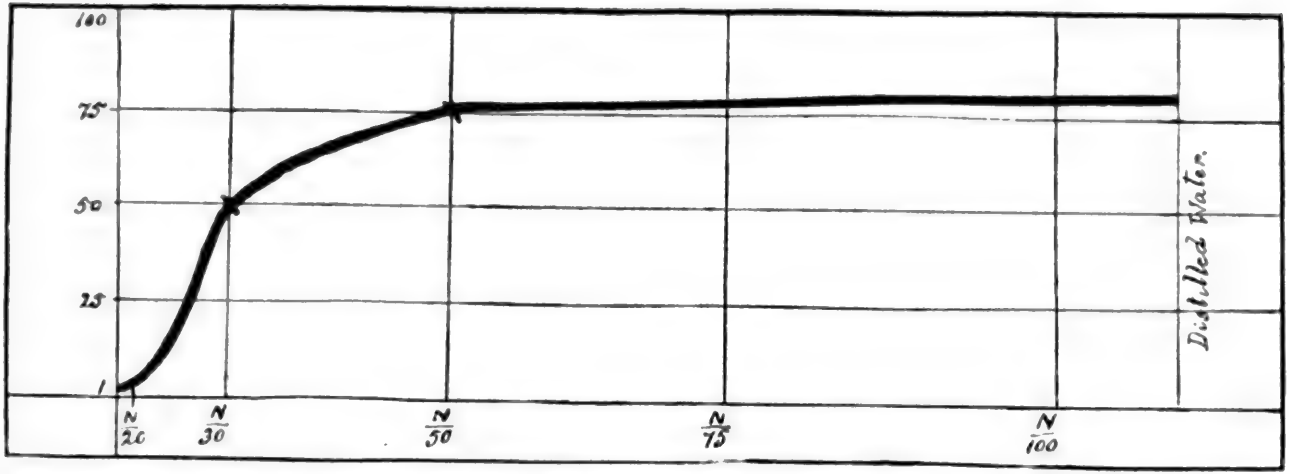

FIG. 1.—The curve of haemolysis of human blood with hyposmotic NaCl solution, the curve being drawn by estimating the amount of haemoglobin in 20 cb. mm. of the supernatant fluid from the centrifuged mixture.

The fact that some of the erythrocytes haemolyse with higher dilutions of saline than others leads to the conclusion that either they are less permeable to water or they can bear the tension of distension from osmosis better than others, and therefore do not rupture so readily. I have, however, already pointed out that osmosis and rupture alone cannot explain the whole phenomenon of haemolysis with hyposmotic saline solutions, and that one has to take the question of mass action into consideration in explaining it[1]. The presence of erythrocytes containing partially discharged haemoglobin among the sediment corpuscles goes against the theory of rupture.

The relation of the amount of haemoglobin in the resistant corpuscles to the total amount in the sample of blood under examination appears to me from observations in health and disease to have an important bearing, and I would suggest that this be called the *relative haemoglobin-value of the resistant erythrocytes.* It may be expressed as the quotient obtained by dividing the amount of haemoglobin in the resistant corpuscles by that of the total blood.

The method by which I estimated the amount of haemoglobin in the resistant corpuscles is described as follows:—In all cases the blood was haemolysed with two parts of $\frac{N}{50}$ saline solution, with which in the case of healthy individuals Wright's haemolytic point is with certainty obtained. After thoroughly mixing 5 cb.mm. of the blood with 10 cb.mm. of $\frac{N}{50}$ saline, the mixture is centrifugalised as thoroughly as possible, and then the sediment is washed several times with $\frac{N}{10}$ saline till the supernatant fluid at the top is perfectly colourless. The sediment is now dissolved in a small quantity of distilled water with the addition of a drop or two of chloroform, and then the amount of haemoglobin is estimated by a Haldane's haemoglobinometer. In those cases in which the amount of haemoglobin in the resistant corpuscles is less than 10 per cent., 10 or 20 cb.mm. of blood is taken and then treated with 20 or 40 cb.mm. of $\frac{N}{50}$ saline respectively, and the amount of haemoglobin in the resistant corpuscles is then estimated. This number divided by two or four, as the case may be, gives the amount of haemoglobin in the resistant corpuscles of 5 cb.mm. of blood.

The accompanying table gives the relative haemoglobin-value of the resistant corpuscles in the blood of some of my students as well as in some cases of anaemia in my wards:—

1. *Loc. cit.*

Table I.—Health

Haemoglobin in 5 cb.mm. of blood	Haemoglobin in the Resistant Corpuscles in 5 cb.mm. of blood	Relative Haemoglobin-value of the Resistant Corpuscles
95	32	0·336
96	40	0·416
96	40	0·416
92	36	0·391
98	35	0·357
110	48	0·436
96	42	0·437
92	34	0·369

Anaemia

Table II.

Haemoglobin in 5 cb.mm. of blood	Haemoglobin in the Resistant Corpuscles in 5 cb.mm. of blood	Relative Haemoglobin-value of the Resistant Corpuscles
40	8	0·200
30	4	0·133
35	10	0·285
36	10	0·277

Table III

Haemoglobin in 5 cb.mm. of blood	Haemoglobin in the Resistant Corpuscles in 5 cb.mm. of blood	Relative Haemoglobin-value of the Resistant erythrocytes
60	21	0·350
46	19	0·413
30	14	0·467
36	15	0·417

Table IV

Haemoglobin in 5 cb.mm. of blood	Haemoglobin in the Resistant Corpuscles in 5 cb.mm. of blood	Relative Haemoglobin-value of the Resistant erythrocytes
35	20	0·571
42	23	0·547

It will be seen from the above tables that while in health the relative haemoglobin-value of the erythrocytes varies within small limits; in anaemia it varies within much wider limits. Thus, in some cases, it is

much below the normal, in others it is almost the same as normal, while in others again it is above the normal. In kala-azar it is generally below the normal, while in ankylostomiasis it is above the normal. The forms of anaemia in which this value is increased or diminished and its clinical significance can only be determined by further investigation.

Permeability of the Erythrocytes to Water as a Factor in the Production of Haemolysis

An explanation may here be offered as to the cause of the differences of the haemoglobin-value of the resistant corpuscles in health and disease. It is possible that the resistant corpuscles are less permeable to water or can bear the tension of distension better than those that haemolyse. This permeability, or the power of resisting rupture, is probably altered in anaemia, being increased in some and diminished in others, while in others again it remains normal.

The entrance of water into the erythrocytes may, therefore, to some extent, be dependent upon their specific permeability, and this may be independent of the force of osmosis. So, also, their power of resisting rupture from distension after the entrance of water into their substance may vary in the different erythrocytes. That these are important factors in the phenomenon of haemolysis is borne out by the following facts:— If one part of human blood is mixed with one part of $\frac{N}{10}$ saline solution and then treated with two parts of $\frac{N}{30}$ saline, we find that the amount of haemolysis is much greater than when the $\frac{N}{10}$ saline contains 1 per cent. formol. The presence of formol cannot in any way change the concentration of the salts in the corpuscles, and its action must result either in increasing the resistance of the erythrocytes to rupture from osmotic distension or diminishing the permeability of water. Similarly, again, when blood is allowed to crenate between the slides for twenty-four hours or more, and then treated with $\frac{N}{10}$ saline soiution, we find that some of them still remain crenated. Now, if crenation were simply due to osmosis, then the corpuscles would swell up and lose their crenation by re-absorption of water when treated with $\frac{N}{10}$ saline solution. The fact that some of them do not lose their crenation shows that they have become less permeable to water. In other words, along with crenation the outer portion of the erythrocytes undergo some changes, as a result

of which they do not allow the free passage of water into their structure by the process of osmosis. The same is also borne out by the fact that crenated corpuscles sometimes occur in the blood in some forms of anaemia. This cannot be due to any stronger concentration of saline in the serum, as in anaemia the chlorides are not much appreciably increased in the serum (see Tables IV and V).

TABLE V

	1	2	3	4	5	6	7	8	Average
Salinity of Normal Serum	0·6435%	0·5850%	0·6435%	0·7020%	0·7020%	0·6435%	0·7020%	0·7020%	=0·6654%

TABLE VI

	1	2	3	4	5	6	Average
Salinity of Serum in Anaemia	0·7605%	0·5850%	0·6727%	0·6727%	0·6435%	0·8772%	= 0·7019%

THE ISOLATION OF THE CONIUM ALKALOIDS FROM ANIMAL TISSUES, AND THE ACTION OF LIVING CELLS AND DECOMPOSING ORGANS ON THESE ALKALOIDS

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The literature on the isolation of the conium alkaloids is very limited, and the only treatises requiring mention are those of Harley[1] and Zalewsky.[2] Zalewsky's method consisted in extracting the organs with acidified water, followed by alcohol, and in shaking out the final residue with petroleum ether after making it alkaline with ammonia. This author has given no quantitative results, and from the fact that he frequently describes scaffold-like crystals as occurring in the final residue, one is inclined to believe that the residue in many cases consisted of ammonium chloride, which gives dense precipitates with phospho-molybdic acid, as he describes.

Preliminary Observations

On account of the volatility of pure coniine, it is impossible to obtain a satisfactory result unless the base is converted into one of its salts which are not volatile at 100° C. The hydrochlorate of coniine is very suitable for the purpose, as it crystallises in long, double-refracting, silky needles, which are easily recognisable. Coming to the estimation of the amount of coniine present, there is a choice of three methods, namely:—(1) The weight of the residue; (2) estimation of pure coniine by titration with normal acid using suitable indicators, such as iodeosin, haematoxylin, cochineal, lacmoid, or congo red; (3) estimation of the alkaloid or its salts by titration with some precipitating reagent, such as Mayer's solution. In the following experiments I have used a combination of the first and third methods, and, in applying the latter, I have estimated the amount of the alkaloid by the number of drops from a capillary tube required to

1. *Old Vegetable Neurotics*, pp. 19 and 80, 1869.
2. *Untersuchungen über das Coniin. Dissert., Dorpat*, p. 17, 1869.

titrate the residue which was acidulated with a definite quantity of dilute hydrochloric acid. This method gave in test cases results which were correct to the fourth decimal place in grams. It will be noted that in many of the experiments the weight of the residue and the amount of alkaloid present, as estimated by Mayer's reagent, do not agree; this is due to the fact that one cannot get a pure alkaloidal residue without going through processes which would entail serious loss of the alkaloid. In such cases the amounts estimated by Mayer's reagent may be taken as correct.

I. Isolation of Coniine by Distillation with an Alkali

The distillation method I have used was that ordinarily employed for such purposes; I have not adopted the method of distilling in hydrogen gas, as I found this unnecessary, also it is not readily applicable to the distillation of animal organs. When coniine hydrochlorate is distilled with sodium hydrate, I have found that a certain amount of ammonia is present in the distillate, and also that the crystals of coniine hydrochlorate re-obtained from the distillate are not nearly so strongly double-refracting as they usually are. If 10 mg. of coniine hydrochlorate is used, the whole of the coniine distils over in the first 12 to 20 c.c. of fluid, and the alkaloid, when estimated by Mayer's reagent, was present to the amount of 9·5 mg. On the other hand, by distilling the coniine hydrochlorate with sodium carbonate, almost no ammonia is produced, and the crystals obtained from the distillate are strongly double-refracting, and, again, when using 10 mg. of the salt the whole of the coniine is contained in the first 20 c.c. of fluid distilled, and this amounted in test cases to exactly 10 mg. Experiments done with sodium bicarbonate showed a more marked production of ammonia but no effect on the refraction of the crystals.

When, however, coniine has to be isolated from organic matters there is always found in the distillate a large amount of ammonia, and, on evaporating the distillate with hydrochloric acid, ammonium chloride forms the greatest proportion of the residue. This salt I have removed by treating the residue with a mixture of two parts of absolute alcohol and one part of ether, or with pure chloroform, which is much more satisfactory. The organic matters were distilled immediately after the addition of the alkaloid.

(1) *Isolation from Urine*

The residue, left after evaporation of the neutralised distillate, is usually so large that it requires to be treated two or three times with the above solvents.

	200 c.c. human urine with 10 mg. coniine hydrochlorate	200 c.c. human urine with 10 mg. coniine hydrochlorate
Residue from chloroform ...	Double-refracting, needle-like crystals	Strongly double-refracting, needle-like crystals and irregular crystals, not double-refracting[1]
Weight of residue ...	0·007 gms. ...	0·013 gms.
Estimated alkaloid by Mayer ...	0·0054 gms. ...	0·0083 gms.
On boiling Mayer's ppt. with sodium-hydrate	Distinct smell of coniine ...	Strong smell of coniine

From the above experiments it will be seen that 83 per cent. of 10 mg. of coniine hydrochlorate can be regained from urine.

(2) *Isolation from Blood*

Forty c.c. of calf's blood with 5 mg. of coniine hydrochlorate was distilled with 100 c.c. of water and excess of sodium carbonate. The crystals of the residue being not quite typical, they were made alkaline by sodium hydrate and the freed base shaken out with ether and re-converted into the hydrochlorate. The crystals obtained were long, transparent, double-refracting needles. A solution of these in acidified water gave dense precipitates with Dragendorff's and Rohrbach's reagents, and also with phospho-wolframic and phospho-molybdic acids, and, on boiling with lime water, a strong smell of coniine was given off. This shows that a sufficient amount of 5 mg. can be regained from 40 c.c. of blood for identification purposes.

(3) *Isolation from Liver*

	100 gms. horse's liver with 10 mg. coniine hydrochlorate	100 gms. horse's liver with 10 mg. coniine hydrochlorate	100 gms. horse's liver with 10 mg. coniine hydrochlorate
Residue from chloroform	Long, needle-shaped crystals, strongly double-refracting	Long, needle-shaped crystals, strongly double-refracting	Pure, long, needle-shaped crystals, strongly double-refracting
Weight of residue ...	0·0045 gms.	0·005 gms.	0·0085 gms.
Estimated alkaloid by Mayer	0·00416 gms.	0·005 gms.	0·0083 gms.
On boiling ppt. with sodium hydrate ...	Strong smell of coniine	Strong smell of coniine	Strong smell of coniine

These results demonstrate that one can obtain back from liver not less than about half and not more than 83 per cent. of the coniine salt.

1. As to what the other crystals present were, one cannot give any opinion. Certainly they did not seem to affect the titration by Mayer's reagent.

(4) *Isolation from Spleen*[1]

	100 gms. horse's spleen with 10 mg. coniine hydrochlorate
Residue from chloroform	Long, transparent, double-refracting needles
Weight of residue	0·0085 gms.
Estimated alkaloid by Mayer	0·00833 gms.
On boiling Mayer's ppt. with sodium hydrate ...	Strong smell of coniine

Thus 83 per cent. of alkaloid can also be regained from spleen.

II. Extraction of Coniine by Treatment with Alcohol and Subsequently Shaking Out with Ether

The finely minced organs containing the alkaloidal hydrochlorate were extracted three times with fresh portions of absolute alcohol. After filtering, the alcohol was evaporated off and the residue again extracted with fresh alcohol; this was filtered, evaporated, and the residue treated with water, any insoluble matter being removed. The watery solution was then made alkaline with sodium hydrate, and the freed base removed by shaking out with ether. In earlier experiments, the ether was evaporated at a low temperature, but in later cases it was acidified with hydrochloric acid before evaporation, in order to obtain the crystalline hydrochlorate. Urine was treated by the same process except that it was evaporated to syrupy consistence with excess of hydrochloric acid, before adding alcohol. When dealing with fatty organs, the ether frequently separated as a muddy layer. This trouble has been avoided either by previous shaking of the still acid water with ether or by evaporating the muddy ether which has separated from the alkaline water with hydrochloric acid, and extracting the residue with water, filtering, and evaporating the watery solution.

(1) *Extraction from Urine*

	100 c.c. human urine with 10 mg. coniine hydrochlorate	100 c.c. human urine with 10 mg. coniine hydrochlorate
Residue from ether	Double-refracting, needle-like crystals and some resinous matter	Double-refracting, needle-like crystals and some resinous matter
Weight of residue	0·010 gms. ...	0·015 gms.
Estimated alkaloids by Mayer ...	0·0046 gms. ...	0·0054 gms.
On boiling Mayer's ppt. with sodium hydrate	No smell of coniine[2] ...	Distinct smell of coniine

1. The minced spleen containing the alkaloid was first treated with tannic acid in presence of HCl to precipitate with albumin as much of the fatty matter as possible. The filtrate was then rather easier to distil. Tannic acid in presence of HCl does not precipitate coniine salts.

2. The probable explanation of this is that since I had been working with pure coniine just before and as the nose becomes rapidly insensible to this smell, I had failed to detect the slight odour which must have been present.

(2) *Extraction from Blood*

Fifty c.c. of calf's blood with 5 mg. coniine hydrochlorate. The blood was first acidified and coagulated by heat, the coagulum was filtered off, and the filtrate evaporated to a syrup and treated as with urine.

Residue from ether.—Long, double-refracting, needle-like crystals, which, when dissolved in acidulated water, gave a dense precipitate with Dragendorff's reagent, but only slight precipitates with phospho-molybdic and phospho-wolframic acids, and Rohrbach's reagent; on boiling the solution with excess of lime water a distinct smell of coniine was evolved.

(3) *Extraction from Liver*

The following table will give some idea of the results which are obtained when the coniine is isolated as a free base. It also shows that by the method of extraction one may isolate substances from liver which show alkaloidal reactions.

	Dragendorff's reagent	Phospho-wolframic acid	Phospho-molybdic acid
Pig's liver 80 gms. without alkaloid	Slight yellow ppt.	Faint white ppt.	Faint yellow ppt.
,, ,, + 10 mg. coniine HCl	Good yellow ppt.	Slight white ppt.	Slight yellow ppt.
,, ,, + 10 mg. coniine HCl	Good yellow ppt.	Faint white ppt.	Faint yellow ppt.

In the following results the ether was acidified before evaporation.

	100 gms. horse's liver with 10 mg. coniine hydrochlorate	100 gms. horse's liver with 10 mg. coniine hydrochlorate
Residue from ether	Transparent, needle-like crystals, slightly double-refracting, along with some resinous matter ...	Long, transparent, double-refracting needles, arranged in sheaves
Weight of residue	0·003 gms. ...	—
Estimated alkaloids by Mayer...	0·0021 gms. ...	0·00125 gms.
On boiling Mayer's ppt. with sodium hydrate	Faint smell of coniine ...	Slight smell of coniine

(4) *Extraction from Spleen*

One hundred grams horse's spleen with 10 mg. coniine hydrochlorate.

Residue.—Long, double-refracting, needle-like crystals with some resinous matter.

Weight of residue.—0·009 grams.

Estimated alkaloid by Mayer.—0·0029 grams.

On boiling Mayer's precipitate with sodium hydrate.—Distinct smell of coniine.

(5) *Extraction from Kidney*

Fifteen grams rabbit's kidney with 10 mg. coniine hydrochlorate.

Residue.—Long, double-refracting needles, which, when dissolved in acid water, gave dense precipitates with alkaloidal reagents and gave off a strong smell of coniine when boiled with lime water.

III. Precipitation of the Alkaloid by Means of Phospho-Wolframic Acid

As phospho-wolframic acid gives precipitates with coniine hydrochlorate in presence of hydrochloric acid up to dilutions of 1 : 10000, the following method was adopted for the isolation of the alkaloids by this means:—The organs containing the alkaloid were coagulated by heat, the coagulum removed, and the filtrate treated with phospho-wolframic acid and some dilute hydrochloric acid till complete precipitation had occurred. The precipitate was filtered off, washed with water containing some phospho-wolframic acid, and then drained free of excess of fluid. The partially dried precipitate was rubbed up in a mortar with excess of barium hydrate and the freed alkaloid extracted with absolute alcohol. After being acidified with hydrochloric acid, the alcohol was evaporated off and the residue extracted with chloroform, filtered, and evaporated, when one ought to obtain the hydrochlorate of coniine pure. Any barium hydrate which dissolves in the alcohol may be removed by means of carbon dioxide or by extracting the residue of chlorides as above with chloroform. Urine was treated directly with the acids, but it is safer to precipitate it twice, since the first precipitate is very copious.

(1) *Precipitation from Urine*

	100 c.c. human urine with 10 mg. coniine hydrochlorate	100 c.c. human urine with 10 mg. coniine hydrochlorate
Residue from chloroform ...	Double-refracting needles ...	Double-refracting needles
Weight of residue	0·008 gms. ...	0·006 gms.
Estimated alkaloid by Mayer ...	0·0042 gms. ...	0·0046 gms.
On boiling Mayer's ppt. with sodium hydrate	Faint smell of coniine ...	Distinct smell of coniine

(2) *Precipitation from Blood*

Fifty c.c. calf's blood with 5 mg. coniine hydrochlorate.

Residue.—Long, needle-like crystals, strongly double-refracting.

Weight of residue.—0·004 grams.

Estimated alkaloid by Mayer.—0·0017 grams.

On boiling Mayer's precipitate with sodium hydrate.—Faint smell of coniine.

(3) *Precipitation from Liver*

	100 gms. horse's liver with 10 mg. coniine hydrochlorate	100 gms. horse's liver with 10 mg. coniine hydrochlorate	100 gms. horse's liver with 10 mg. coniine hydrochlorate
Residue from chloroform	Long, needle-shaped crystals in sheaves; strongly double-refracting	Double-refracting, needle-like crystals	Few, irregular, double-refracting crystals and some fatty matter
Weight of residue ...	—	0·008 gms.	0·005 gms.
Estimated alkaloid by Mayer	0·0037 gms.	0·0025 gms.	0·0005 gms.[1]
On boiling Mayer's ppt. with sodium hydrate	Distinct smell of coniine	Smell of coniine	Faint, but recognisable smell of coniine

(4) *Precipitation from Spleen*

One hundred grams horse's spleen with 10 mg. coniine hydrochlorate.

Residue from chloroform.—Long, double-refracting, needle-like crystals.

Weight of residue.—0·008 grams.

Estimated alkaloid by Mayer.—0·0021 grams.

On boiling Mayer's precipitate with sodium hydrate.—Distinct smell of coniine.

(5) *Precipitation from Kidney*

Fifteen grams rabbit's kidney with 10 mg. coniine hydrochlorate.

Residue from chloroform.—Double-refracting, needle-like crystals which, dissolved in acid water, gave dense precipitates with alkaloidal reagents and gave off a strong smell of coniine when boiled with lime water.

IV. Precipitation by Kraut's Reagent

This process proved in my hands quite useless for coniine, as the precipitate was of such a nature that it passed readily through a filter, and, again, it was found impossible to get rid of the iodine completely.

Conclusions on the Isolation of Coniine

It will be observed that, in the case of the distillation, the best results are in three cases 83 per cent., while with the alcohol and ether process and precipitation method the results vary considerably, and in most instances the results are considerably below 50 per cent. If one makes an average of all the figures one finds that the average return by distillation is 65·7 per cent., while the other two methods only show half

1. This liver proved very difficult to filter after boiling.

this amount. Again, the distillation method can be carried through in at most three hours, while the other two occupy eight hours at least, and the alkaloidal salt obtained in the end is much purer in the case of distillation, as may be surmised by the small differences between the weights of the residues and the amounts of alkaloid found by Mayer's reagent. Taking everything into account, only one conclusion is possible, that, for practical purposes and satisfactory results, distillation is the most valuable method for isolating coniine from tissues.

Action of Living Cells on Coniine

In order to ascertain whether living cells had any power of decomposing coniine or in any way interfering with its recognition in the animal organism after death, finely minced liver containing coniine hydrochlorate was mixed with 100 c.c. normal saline solution, to which had been added 1 c.c. of chloroform and 1 c.c. of toluol to prevent decomposition, and the whole left in a water bath at a temperature of 38° C. for a definite period. The alkaloid was re-isolated by distillation.

	60 gms. rabbit's liver with 20 mg. coniine hydrochlorate	60 gms. rabbit's liver with 10 mg. coniine hydrochlorate	80 gms. rabbit's liver with 10 mg. coniine hydrochlorate	100 gms. horse's liver with 10 mg. coniine hydrochlorate	100 gms. horse's liver with 10 mg. coniine hydrochlorate
Time on water bath ...	16 hrs.	18 hrs.	24 hrs.	12 hrs.	18 hrs.
Residue from chloroform	Long, double-refracting, needle-like crystals	Long needles, slightly double-refracting	Some long, double-refracting needles	A few needle-like crystals, not double-refracting	A few long, double-refracting crystals
Weight of residue ...	0·0065 gms.	0·003 gms.	0·010 gms.	0·002 gms.	0·002 gms.
Estimated alkaloid by Mayer	0·0065 gms.	0·0021 gms.	0·0037 gms.	No ppt.	0·0004 gms.
Boiling ppt. with sodium hydrate ...	Distinct smell of coniine	Faint smell of coniine	Distinct smell of coniine	No smell	Faint smell of coniine

All these livers were re-distilled with dilute sulphuric acid, the distillates neutralised with sodium carbonate and evaporated to dryness. To the residue absolute alcohol and excess of concentrated sulphuric acid were added, and in all the above cases a very strong smell of butyric ether was evolved, mixed in some cases with the smell of acetic ether. A test experiment, done with liver alone, gave a distinct smell of butyric acid, but not so powerful as that of the above cases.

Conclusions.—If one takes the average amount of alkaloid which may be regained from liver by distillation as 58 per cent.—a low estimate—it will be seen from the table that there is a remarkable loss of alkaloid

after the cells of the liver have been allowed to act on it even for a short time. The powerful smell of butyric acid would seem to suggest that the coniine may probably be broken up into this substance. Whether this suggestion is correct or not, I am unable as yet to say, but I hope to follow this up shortly.

Action of Decomposing Tissues on Coniine

To ascertain whether decomposing tissues had any influence on coniine, minced liver with the alkaloidal hydrochlorate was mixed with 100 c.c. of normal saline solution and left in a water bath at 38° C. for a definite period. The alkaloid was isolated by distillation.

	60 gms. rabbit's liver with 10 mg. coniine hydrochlorate	160 gms. horse's liver with 10 mg. coniine hydrochlorate	100 gms. horse's liver with 10 mg. coniine hydrochlorate	100 gms. horse's liver with 10 mg. coniine hydrochlorate	100 gms. horse's liver with 10 mg. coniine hydrochlorate
Time on water bath ...	18 hrs.	24 hrs.	24 hrs.	24 hrs.	24 hrs.
Residue from chloroform	Some transparent double-refracting crystals, which were very deliquescent	Double-refracting needles, not typical of coniine hydrochlorate, and very deliquescent	Deliquescent needles, some double-refracting, others not	Needle-like crystals, not double-refracting and not deliquescent	Double-refracting needles, not typical of coniine hydrochlorate, and very deliquescent
Weight of residue ...	0·009 gms.	0·004 gms.	0·01 gms.	0·003 gms.	0·009 gms.
Estimated alkaloid by Mayer	0·005 gms.	0·0021 gms.	0·0025 gms.	0·0021 gms.	0·0008 gms.
Boiling ppt. with sodium hydrate ...	—[2]	Peculiar smell, resembling ammonia	—[1]	Peculiar smell, resembling ammonia	Strong and peculiar smell, resembling ammonia

Conclusions.—It will be apparent that the maximum amount of alkaloid found after twenty-four hours was 2·5 mg., but the crystals of the residue were only partially double-refracting and, at the same time, very deliquescent, while the smells evolved in all cases were ammonia-like. In Experiment III it should have been easily possible to obtain the uranium nitrate reaction for coniine, but this failed; in Experiment I, however, a positive reaction was got, probably since the quantity of liver was small and the time for decomposition short. I am, therefore, of the opinion that no coniine was present in the distillate from the latter four experiments, but that the substance obtained was a body possessing

1. On treating the Mayer's precipitate in this case with sodium carbonate, carbon bisulphide, and uranium nitrate, and shaking up with toluol—no red colour was obtained in the liquid.

2. The same reaction in this case, however, gave a red colour in the toluol which would indicate that coniine was present.

some of the characters of coniine hydrochlorate, while lacking others. The suggestion is possible that many of these crystals were cholin hydrochlorate, which is double-refracting and very deliquescent. This salt also crystallises in needles, and when boiled with sodium hydrate a smell of trimethylamine is given off which would correspond closely to the smell perceived above. The cholin hydrochlorate must have passed through the filter in a deliquesced state along with the chloroform extract.

Isolation of Conhydrine

The isolation of conhydrine proved more difficult, on account of the fact that it cannot be distilled from watery solutions, even when these are saturated with calcium chloride or under pressures as low as 10 mm. of mercury. Conhydrine is also less soluble in ether than in water, and chloroform, in which it is readily soluble, gave unsatisfactory results, so that it was discarded in favour of repeated shakings with fresh ether. Again, conhydrine forms a very deliquescent hydrochlorate, and was therefore isolated as the free base, care being taken to evaporate solutions of this at low temperatures. With reference to the process of precipitation, phospho-wolframic acid is again the only suitable method, but its delicacy only reaches to dilutions of 1 : 1000. The extracts of the organs were therefore evaporated to small bulk before adding the reagent, and urine was first precipitated with lead acetate in presence of hydrochloric acid, in order to avoid bulky precipitates with the reagent. For the estimation of the amount of alkaloid recovered, phospho-wolframic acid was substituted for Mayer's reagent, as the latter is not sufficiently delicate.

(1) *Extraction from Urine by Alcohol and Ether*

	50 c.c. human urine with 10 mg. conhydrine	100 c.c. human urine with 10 mg. conhydrine	100 c.c. human urine with 10 mg. conhydrine	100 c.c. human urine with 10 mg. conhydrine	200 c.c. human urine with 10 mg. conhydrine
Residue from ether ...	Double-refracting needles and plates, the latter having one angle cut out	Needle-like, double-refracting crystals	Double-refracting, needle-like crystals	Double-refracting needles and flat, oblong crystals, having an angle cut out	Double-refracting, needle and plate-like crystals, the latter having an angle cut out
Weight of residue	0·008 gms.	0·005 gms.	0·012 gms.	0·015 gms.	0·014 gms.
Estimated alkaloid by phosp.-wolf. acid ...	0·0012 gms.	0·0017 gms.	0·0045 gms.	0·0075 gms.	0·0037 gms.
On boiling ppt. with sodium hydrate ...	Distinct smell of conhydrine	Distinct smell of conhydrine	Strong smell of conhydrine	Very strong smell of conhydrine	Strong smell of conhydrine

(2) *Extraction from Liver by Alcohol and Ether*

	100 gms. horse's liver with 10 mg. conhydrine	100 gms. horse's liver with 10 mg. conhydrine	100 gms. horse's liver with 10 mg. conhydrine
Residue from ether ...	No crystals	Needle-like crystals, slightly double-refracting	Double-refracting crystals of oblong form with one of the angles cut out
Weight of residue ...	0·002 gms.	0·003 gms.	0·009 gms.
Estimated alkaloid by phosp.-wolf. acid ...	0·0005 gms.	0·00075 gms.	0·0037 gms.
On boiling ppt. with sodium hydrate ...	Faint smell of conhydrine	Distinct smell of conhydrine	Strong smell of conhydrine

(1) *Precipitation from Urine by Phospho-wolframic Acid*

	100 c.c. dog's urine with 10 mg. conhydrine	100 c.c. human urine with 10 mg. conhydrine	100 c.c. human urine with 10 mg. conhydrine
Residue from chloroform	Colourless, imperfect, double-refracting crystals	Imperfect, double-refracting crystals	Irregular, double-refracting crystals
Weight of residue ...	0·004 gms.	0·006 gms.	0·009 gms.
Estimated alkaloid by phosp.-wolf. acid ...	0·0015 gms.	0·0025 gms.	0·006 gms.
On boiling ppt. with sodium hydrate ...	Faint smell of conhydrine	Distinct smell of conhydrine	Strong smell of conhydrine

(2) *Precipitation from Liver by Phospho-wolframic Acid*

	100 gms. horse's liver with 10 mg. conhydrine	100 gms. horse's liver with 10 mg. conhydrine	100 gms. cow's liver with 10 mg. conhydrine
Residue from chloroform	Irregular, double-refracting crystals	No crystals	Transparent needles, double-refracting and very deliquescent
Weight of residue ...	0·004 gms.	0·003 gms.	0·005 gms.
Estimated alkaloid by phosp.-wolf. acid ...	0·0012 gms.	0·0025 gms.	0·0025 gms.
On boiling ppt. with sodium hydrate ...	Distinct smell of conhydrine	Distinct smell of conhydrine	Strong smell of conhydrine

Conclusions on the Isolation of Conhydrine

The figures given above show that, by neither of these two methods are results got which are in any way constant. The higher figures are those obtained from later experiments and show probably about the limit attainable by these processes. One can, however, say that conhydrine can be isolated from both urine and liver in quite appreciable amounts, even when the quantity added consists of only 10 mg. in 100 c.c. or grams of substance.

Action of Living Cells on Conhydrine

The method used was the same as that described for coniine. The alkaloid was extracted by alcohol and ether.

	100 gms. cow's liver with 10 mg. conhydrine	100 gms. horse's liver with 10 mg. conhydrine	100 gms. horse's liver with 10 mg. conhydrine
Time on water bath ...	18 hrs.	18 hrs.	12 hrs.
Residue from ether ...	Thin gummy layer, no crystals	No crystals	A few double-refracting needle-like crystals
Weight of residue ...	0·011 gms.	0·003 gms.	0·012 gms.
Estimated alkaloid by phosp.-wolf. acid ...	0·00076 gms.	0·001 gms.	0·0037 gms.
On boiling ppt. with sodium hydrate ...	Doubtful smell of conhydrine	Faint smell of conhydrine	Distinct smell of conhydrine

Action of Decomposing Tissues on Conhydrine

The alkaloid was treated in the way described for coniine and isolated by means of the alcohol and ether process.

	100 gms. horse's liver with 10 mg. conhydrine	100 gms, horse's liver with 10 mg. conhydrine
Time on water bath ...	18 hrs.	18 hrs.
Residue from ether ...	Gummy skin, no crystals ...	Resinous residue and some double-refracting, oblong crystals
Weight of residue ...	0·008 gms. ...	0·008 gms.
Estimated alkaloid by phosp.-wolf. acid ...	0·0015 gms. ...	0·002 gms.
On boiling ppt. with sodium hydrate ...	Faint smell of conhydrine ...	Distinct smell of conhydrine

Conclusions. –The above results show that, on account of the fact that the method of isolation does not give constant returns, it is impossible to draw any definite conclusion as to whether or not conhydrine is affected by the action of living cells or decomposed tissues.

Isolation of Pseudo-conhydrine

On account of the small amount of this alkaloid which I possess, I have limited this research to a very few experiments, which, however, show fairly satisfactory results. The difficulties encountered in the isolation of pseudo-conhydrine are the same as those detailed under conhydrine, and they were avoided by the same methods. Pseudo-conhydrine cannot be distilled from watery solutions.

(1) *Extraction from Urine by Alcohol and Ether*

	60 c.c. human urine with 10 mg. pseudo-conhydrine	100 c.c. human urine with 10 mg. pseudo-conhydrine	200 c.c. human urine with 10 mg. pseudo-conhydrine
Residue from ether ...	Double-refracting, needle-like crystals	Double-refracting, small, needle-like crystals	Double-refracting, small, needle-like crystals
Weight of residue ...	0·014 gms.	0·006 gms.	0·007 gms.
Estimated alkaloid by phosp.-wolf. acid ...	0·0062 gms.	0·0035 gms.	0·0017 gms.
On boiling ppt. with sodium hydrate ...	Strong smell of pseudo-conhydrine	Distinct smell of pseudo-conhydrine	Faint smell of pseudo-conhydrine

(2) *Extraction from Liver by Alcohol and Ether*

	100 gms. horse's liver with 10 mg. pseudo-conhydrine	100 gms. horse's liver with 10 mg. pseudo-conhydrine
Residue from ether	No distinct crystals ...	Small hair or needle-like crystals, double-refracting
Weight of residue	0·002 gms. ...	0·007 gms.
Estimated alkaloid by phos.-wolf. acid	0·0005 gms.[1] ...	0·004 gms.
On boiling ppt. with sodium hydrate	No smell of alkaloid ...	Distinct smell of pseudo-conhydrine

(1) *Precipitation from Liver by Phospho-wolframic Acid*

100 grams horse's liver with 10 mg. pseudo-conhydrine.

Residue from chloroform.—No crystals.

Weight of residue.—0·002 grams.

Estimated alkaloid by phospho-wolframic acid.—0·0015 grams.

On boiling precipitate with sodium hydrate.—A faint smell of pseudo-conhydrine.

Conclusions.—The results indicate that, with urine, it is possible to isolate 35 per cent. of the alkaloid added, while, with liver, about 40 per cent. can be regained. The single experiment done with phospho-wolframic acid shows a fairly good return for that method, namely, 15 per cent.

Action of Decomposing Tissues on Pseudo-conhydrine

One experiment was done with this alkaloid by leaving 10 mg. in 100 grams of horse's liver in a water bath for twelve hours, as described for coniine. The alkaloid was extracted by alcohol and ether.

Residue from ether.—A few, irregular, double-refracting crystals in a gummy matrix.

1. Liver difficult to filter.

Weight of residue.—0·003 grams.

Estimated alkaloid by phospho-wolframic acid.—0·0007 grams.

On boiling precipitate with sodium hydrate.—No smell which resembled pseudo-conhydrine.

No definite conclusion can be drawn from this, since, in one case, as small a quantity was regained from fresh liver, although that was due to difficulties in carrying out the process.

Summary

1. The most satisfactory method for the isolation of coniine from animal tissues is that of distillation.

2. Coniine appears to be decomposed both by the action of living cells and by decomposing tissues.

3. Conhydrine and pseudo-conhydrine can be isolated from animal tissues by extraction with alcohol and by precipitation with phospho-wolframic acid, but these methods do not give sufficiently constant results to allow of any definite conclusions being drawn as to the action of living cells or decomposing tissues on these poisons.

I have, finally, to thank Professor Kobert of Rostock for procuring for me the materials used in this research and for his kind assistance and advice. I wish also to acknowledge my indebtedness to Professor MacWilliam and Professor Cash for their courtesy in advising me with regard to the arrangement of the results.

SOME OBSERVATIONS UPON THE ERROR IN THE OPSONIC TECHNIQUE[1]

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Introduction

Anyone who has compared the figures obtained for the opsonic index of the same serum as estimated quite independently by two observers, will be aware that the difference between their results is often considerable even after extensive experience of opsonic technique, and when the precaution has been taken of enumerating many leucocytes.

We have recently calculated a large number of indices three times, employing tubercle bacilli and staphylococci, and consider that a detailed account of the experimental errors in our own work, together with a résumé of the errors obtained by other workers, may be of some interest to those engaged in this line of research. There also arises the larger question: is the Wright technique, even including all its most recent modifications, so hopelessly inaccurate that no deductions whatever can be drawn from it?

In a paper at present in the press[2] we have detailed the results of calculating eighty consecutive indices, three times, i.e., 240, with staphylococcus, and forty consecutive indices, three times, i.e., 120 with tubercle.

The staphylococcus indices were calculated on seventeen different days, and the tubercle indices on ten different days, almost invariably twelve indices, or four sets of three, on each day.

The technique of Wright and Douglas was adopted.

One half of the indices calculated were the result of comparing the degree of phagocytosis obtained with *different* sera, but the *same* leucocytes, i.e., they were opsonic indices; the other half the result of comparing the degree of phagocytosis obtained with *different* strains of leucocytes, but the *same* sera; these we have called 'Cytophagic Indices.'

1. The greater part of this paper formed a portion of a Thesis for the degree of M.D. Cantab.
2. *Journal of Pathology and Bacteriology*, Vol. XIV, No. 1.

According to the Wright School, the latter indices should always be unity, because the 'phagocytic power of corpuscles from different sources' is 'the same.'[1] We have demonstrated that the inherent phagocytic power of corpuscles is not always the same, and the cytophagic indices in our series of observations vary from about 1·2 to about 1·7.

This fact, however, does not affect the present question, viz., the accuracy of the Wright technique for measuring phagocytosis, and we have included all our figures, both of opsonic and of cytophagic indices, in order to increase the number of observations available for statistical purposes.

The Method by which the indices were calculated is briefly as follows:— A sample of serum and of corpuscles were drawn from three normal men, 'G,' 'L,' and 'A,' i.e., three samples of each were prepared. Twelve separate phagocytic mixtures were put up from these, and the counts obtained enabled us to calculate twelve indices, three opsonic indices for 'A' and for 'L' respectively, and three cytophagic indices for 'A' and for 'L' respectively; the washed leucocytes and serum of 'G' furnishing the control.

The method by which these indices were calculated from the various combinations of sera and leucocytes is tabulated below.

TABLE I, GIVING THE VARIOUS COMBINATIONS OF WASHED LEUCOCYTES OF 'G,' 'L,' AND 'A,' USED IN THE PHAGOCYTIC MIXTURES, AND THE METHODS OF CALCULATING THE INDICES, 'G'S' SERUM OR LEUCOCYTES BEING USED AS 'CONTROL'

Source of Serum in phagocytic mixture		Source of Leucocytes in phagocytic mixture		Number of phagocytic mixture
A.	...	A. } A. }	...	1.
A.	...	G.	...	2.
A.	...	L.	...	3.
G.	...	A.	...	4.
G.	...	G. } G. }	...	5.
G.	...	L.	...	6.
L.	...	A.	...	7.
L.	...	G.	...	8.
L.	...	L. } L. }	...	9.

Fractions used for the calculation of 'A's' three opsonic and three cytophagic indices:—

A's opsonic indices	...	[illegible]	[illegible]	[illegible]
A's cytophagic indices	...	[illegible]	[illegible]	[illegible]

1. *Practitioner*, May, 1908.

The same principle was adopted in the calculations of 'L's' indices. It is clear that 'A's' three opsonic indices were obtained from six distinct phagocytic mixtures; therefore, any difference between the three indices is due not only to errors from counting, but also to errors in 'putting up' the phagocytic mixture, preparing and staining the films. This is a more thorough and practical way of ascertaining the degree of error inherent in Wright's technique than that of recounting the same slide—a method adopted by some observers.

It will be noticed that the counts obtained from phagocytic mixtures 1, 5, and 9 are really the mean of two separate estimations. This tends to increase the accuracy of our indices somewhat, and gives us a slight advantage in comparing our errors with those of other writers. The same holds good for the other sets of triple indices. The reasons for this somewhat complicated method of calculating the indices, together with an example fully worked out with figures, are given elsewhere.

The bacterial emulsion was prepared as recently recommended by Fleming.[1]

It is important to note that the washed erythrocytes of these three individuals were not agglutinated by any of the combinations of sera employed.

Fleming[2] who recently contributed a valuable paper on the accuracy of the opsonic technique from Wright's[3] laboratory, states that 'a diminution in the number of washed corpuscles in the opsonic mixture causes an increased amount of phagocytosis.' In order, therefore, to eliminate errors from this source and ensure that the relative amounts of normal salt solution and washed corpuscles taken up in the opsonic pipettes were the same in all the triple estimations, the tubes containing them were placed vertically between the palms of the hand and vigorously rolled to and fro immediately before a quantum was removed, thus the corpuscles and salt solution were always well mixed.

Incubation.—The phagocytic mixtures, consisting of equal parts of corpuscles, serum and bacterial emulsion in salt solution, were placed for fifteen or twenty minutes in a patent incubator at 37° C. This apparatus consists essentially of a metal box filled with water, into one side of which two horizontal and parallel rows of narrow metal tubes are inserted. Each tube is open to the air in front, but is surrounded by water which is maintained at a constant temperature by a single flame of gas.

1. *Practitioner*, May, 1908.
2. Fleming, *Practitioner*, May, 1908, p. 618.
3. Wright, *Lancet*, 1907.

It was found, even when the apparatus was most carefully regulated, that the temperature of the tubes in the centre of the upper row was liable to be half a degree C. or more higher than in the periphery of the lower row, on account of deficient circulation of the water.

This defect was completely remedied by constantly agitating the water in the incubator with a rotating paddle. We do not know whether a difference of $\frac{1}{2}$° C. between the individual tubes will exert any appreciable effect upon the amount of phagocytosis, but it is advisable that all scientific apparatus should be as perfect as possible, especially in the technique so full of pitfalls as the opsonic technique. The readings of temperature were taken with a microscope from a thermometer graduated in tenths of a degree C. After incubation the contents of the pipettes were remixed and three films prepared from each one.

The smears were made in the usual way by placing the drop at one end of the slide and drawing it out with another narrower slide held at an angle, great care being taken to make the termination of the smear as rectangular as possible. (Vide diagram.)

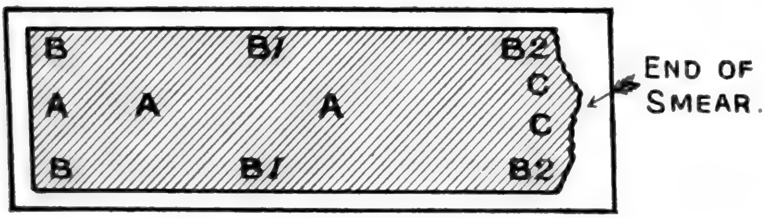


DIAGRAM OF SLIDE WITH BLOOD SMEAR.

Staining.- The staphylococcus films were stained by Leishman's method, using accurately measured quantities of stain and distilled water, and rocking the slides at intervals to ensure uniform staining—a very important point. The tubercle films were fixed in saturated corrosive sublimate solution, and stained by pouring upon them boiling carbol fuschin, decolourised in 2 per cent. sulphuric acid, washed with 5 per cent acetic acid, and counter-stained in methylene blue.

Counting. The number of bacteria phagocytosed by sixty polymorphonuclear leucocytes were enumerated in two out of the three films made from each phagocytic mixture. Thirty cells were taken from the two borders beginning at the corners B 2 and passing backwards to B 1. Thus the *same part* of every film was examined. All the eosinophiles and all polymorphs with indistinct edges owing to damaged cytoplasm were

eliminated from the counts. Clumps of more than three leucocytes were also omitted.

When the batch of films was finished, the figures were added up, and if there was a deviation from the arithmetic mean of the counts of any two duplicate films of more than 5 per cent., another sixty leucocytes were taken from the third uncounted film and an average struck from the three sets of figures. Half the total number of leucocytes counted (120) were selected from one film and half from another, in order to diminish the errors due to improper smearing, unequal staining, or inaccurate counting, and, lastly, because it is obviously advantageous, when there is a marked difference between the figures obtained from the two films, to count an additional sixty from the third film.

If the figures when added up were contrary to expectation, recounts were not made, nor were an extra sixty cells counted in the hope that 'all might be well.' In fact, no recounts were made except in the case of some half dozen slides, because the counter was interrupted or found his attention wandering. No counts were discarded on the grounds that the films were improperly stained. The usual number of polymorphonuclear leucocytes counted was sixty, but occasionally eighty or fifty, according to the strength of the emulsion. The number once fixed by an examination of the first film was invariably the same for all the batch. As duplicate films were used, it is clear that the total number of cells counted from each phagocytic mixture was usually 120, sometimes 180 or 100.

Elimination of Auto-suggestion.—In order to eliminate any auto-suggestion in counting, all the stained films, usually twenty-four in all, were placed in numbered compartments, the figures upon them being rubbed off. An attendant handed these unnumbered films indiscriminately to the observer, who thus remained ignorant of which films he was counting.

Every film was counted by the same individual, E. E. G.

Statements of the Wright School Regarding Errors of Technique

Three definite statements have been made by the Wright School regarding the accuracy of the method.

1. In 1907 Wright[1] said the error 'in the case of normal bloods in the hands of a good worker' is 'rarely greater than plus or minus 5 per cent.'

1. *Lancet*, p. 427, August 17, 1907.

2. Next year Fleming,[1] who had carefully investigated the matter, concluded that 'duplicate estimations of the tuberculo-opsonic index of tuberculous patients can be performed, the results differing from each other by less than 20 per cent., except in rare instances (two in fifty-two)'; that is to say, he admits that the limits of error are at least twice as wide as those laid down by Wright.

We have examined the figures given on Table VIII, in which thirty-eight duplicate indices are calculated by two observers putting up separate opsonic mixtures; fourteen pairs were from normal and twenty-four pairs (not twenty-six as stated by Fleming) from tuberculous persons. We find that the average difference between the fourteen pairs of normal indices is 0·076, the maximum being 0·29, and between the twenty-four pairs of tuberculous indices 0·068, the maxima being 0·21 and 0·20. The average difference between the whole thirty-eight pairs is 0·071; nine out of these, or 25 per cent., differed by more than 10 per cent., and two, or 5 per cent., by more than 20 per cent.

3. Fleming stated in 1908 that 'two practised observers can count same slides and obtain results in almost all cases within 10 per cent.' Analysing the figures in Fleming's table, VII, we find that the average difference in forty-one indices obtained by two observers A and B counting the same slides works out at 0·064 per cent. Seven of these differed by more than 10 per cent., the maximum difference being 20 per cent.

The comparison of counts from the *same* slides is, however, a very unsatisfactory way of testing the accuracy of the method, for all errors associated with putting up the phagocytic mixtures are excluded.

Methods of Calculating Errors

The errors in this paper have been estimated in three ways.

Method A.—We have calculated the percentage deviation from the arithmetic mean of the two counts of duplicate slides made from the same phagocytic mixture. It has already been mentioned that usually sixty cells were counted on each slide, and that if there was a deviation from the arithmetic mean of more than 5 per cent. another set of sixty cells was counted from the third slide.

Method B.—The simplest and most practical way of estimating the degree of error in opsonic work is to compare the difference between indices calculated from the duplicate phagocytic mixtures. We have already alluded to Fleming's paper, where the average difference between

1. *Practitioner*, p. 634, May, 1908.

thirty-eight pairs of indices calculated by two observers from duplicate phagocytic mixtures is 0·07.

In our paper three sets of indices have been calculated from triplicate phagocytic mixtures; in order, therefore, to compare our results with those of Fleming we have taken the mean of the difference between each of the three arranged in pairs. For example, on July 20th the three indices of A were:—(1) 0·778, (2) 0·889, (3) 0·825. The difference between 1 and 2 is 0·11, between 1 and 3 is 0·05, and between 2 and 3 is 0·06. Now, 0·11 + 0·05 + 0·06 = 0·22. The mean of the differences of the three pairs is, therefore, 0·07.

Our error so calculated is 0·17 for all duplicate *staphylococcus* indices and 0·15 for all duplicate *tubercle* indices, which compares rather unfavourably with Fleming's figures of 0·07 for tubercle, though it is within his 20 per cent. limit.

As previously mentioned, half our indices were opsonic, the other half we have called cytophagic; that is to say, they were a comparison of the phagocytic power of different strains of leucocytes put up with the *same* serum. For some reason, probably accidental, the error in the cytophagic indices, calculated by *Method B*, is slightly less than in the opsonic. The figures being—staphylococcus, 0·14 and 0·20, and for tubercle, 0·14 and 0·15 respectively.

Method C.—Errors have been estimated in another way also, by taking the maximum deviation from the mean of the triple estimation. For example, the mean of the three indices 0·778, 0·889, and 0·825 is 0·831. The maximum deviation from the mean is, therefore, in this case 0·058.

The application of this method to Fleming's figures would give 0·035 for the average maximum deviation for the mean.

These methods of estimating the error of technique may be rather elementary for a statistician, but they have the merit of being easily understood by the non-mathematical mind. *Method B* is the most valuable; it deals with indices, not counts, gives the most consistent results, and can most easily be compared with duplicate estimations of other workers.

TABLE I, SHOWING AVERAGE DIFFERENCE BETWEEN DUPLICATE AND TRIPLICATE INDICES OF THE SAME SERUM BUT FROM SEPARATE PHAGOCYTIC MIXTURES AS CALCULATED FROM THE RESULTS OF DIFFERENT OBSERVERS

Paper by	Observer	Average difference	No. of indices preceding figures based on	Approximate number of cells counted	Approximate number of bacteria per cell	Organism
Fleming	Two	0·07	76	100	2*	Tubercle
Hort†	'B'	0·10	6	100	2, 3	„
„	'O'	0·12	6	100	2·0	„
„	'T'	0·10	6	100	2·3	„
E. G.	E. G.	0·15	120	120	3·2	„
E. G.	E. G.	0·17	240	120	3·5	Staphylococcus
Strangeways‡	F. G. & W.	0·36, 0·45	36	50	1·2	Tubercle
„	W.	0·36, 0·40	18	50	1·2	„
„	F. G.	0·51, 0·55	18	50	1·2	„

* NOTE.—The exact strength of emulsion employed by Fleming's workers (in Table VIII) cannot be ascertained, but we assume it is similar to that in Table VII, viz., approximately 2. *Practitioner*, pp. 630, 633, May, 1908.

† See page 313. ‡ See page 309, Table III.

VARIOUS UNFAVOURABLE OPINIONS UPON THE ACCURACY OF THE OPSONIC TECHNIQUE

Many writers, especially in America, have recently impugned the accuracy of the opsonic technique.

After working at the tuberculo-opsonic index for several months and eliminating clumping and fragmentation of the bacilli in the emulsion by exposing their culture to direct sunlight, Jeans and Sellards[1] conclude that 'the limits of error in our technique, at least, are so great as to render the method inapplicable for clinical work.' It may be added that they followed the method of Wright as closely as possible.

Moss,[2] who superintended very extensive comparative tests carried out by three observers simultaneously, comparing the percentage and greatest percentage variations from different counts, concludes that 'none of the present methods of estimating the opsonic content of the blood seem sufficiently accurate to be of practical value.'

Dr. Bolduan,[3] of the Department of Health of the City of New York, found that, 'for reasons not yet understood, duplicate and triplicate tests made on the same serum at the same time and under apparently identical conditions often yield widely divergent results.'

Simon[4] speaks of the 'phantastic curves' and 'absolutely absurd' results which Wright's index sometimes gives.

Thomas[5] remarks 'that, aside from technical difficulties, the question

1. *Bulletin of the Johns Hopkins Hospital*, p. 234, June and July, 1907.
2. *Bulletin of the Johns Hopkins Hospital*, p. 234, June and July, 1907.
3. *Long Island Medical Journal*, Vol. I, No. 10, p. 6.
4. *Journal of Experimental Medicine*, Vol. IX, No. 5, pp. 488, 489.
5. *Journal of American Medical Association*, p. 1249, October 12, 1907.

of personal equation evolved in opsonic determinations is so serious as to practically nullify the value of the method in most instances.'

Potter[1] concludes that Wright's method of estimating the opsonic indices in bacterial infection is hardly accurate enough to compensate for the amount of time involved in its application.

Jügens,[2] discussing the well-known difficulty of phagocytosis of clumped bacilli, quotes an example where an index was raised from 0·95 to 1·13 by the inclusion of two out of one hundred leucocytes which had ingested twenty-seven and twenty-nine cocci respectively.

Work of Fitzgerald, Whiteman and Strangeways.—A valuable enquiry into the accuracy of the opsonic index was undertaken by Fitzgerald, Whiteman and Strangeways,[3] in 1907, on account of 'the very unsatisfactory and discordant results' obtained in the pathological laboratories of Oxford and Cambridge Universities. One of the investigators (Whiteman) had received instruction in the technique at St. Mary's Hospital, London, and had been engaged for a year in the estimation of indices, and was under the impression that 'the error in his work was seldom greater than 10 per cent.'

In the first part of their research they made counts and estimated indices from phagocytic mixtures and smears prepared by themselves, and obtained very unfavourable results. The most important of all the statistics are those in Table IV, page 124, which give various opsonic indices obtained by two observers, who put up the opsonic mixture and calculated the indices of the same sera absolutely independently.

Two capsules of serum were taken daily from a tuberculous patient, viz., R.1 and R.2, and from two normal persons used as controls, viz., W.1 and W.2 and F.G.1 and F.G.2. They were numbered by a disinterested observer S. to eliminate the unconscious influence on the results of knowing which blood was being dealt with. As a rule these observers F.G. and W. put up the blood within a short time of each other; the washed corpuscles were usually taken from the same individual F.G. For the sake of simplicity we have modified the headings in Table IV and have not quoted all the indices calculated from the various combinations of sera, including those by using S.'s control, and have added columns showing (1) the variations between the duplicate indices of different observers, and (2) the variations between the duplicate indices of the same

1. *Journal of American Medical Association*, p. 1815, November 30, 1907.
2. *Berliner Klinischer Wochenschrift*, p. 641, May 30, 1908.
3. *Bulletin for the Study of Special Diseases*, Cambridge, Vol. I, No. 8.

observers. The sera from R.W. and F.G. were divided into two capsules for purposes of convenience, so all four indices on February 4th, for example, in which W.'s serum was taken as control, should be theoretically the same.

Table III, Showing Variation in the Opsonic Index Obtained in Comparison of the Same Sera by Different Observers

Date 1907	Opsonic mixtures put up and indices calculated by F. G. — Capsules of serum from which indices calculated. Patient	Control	Index	Variations in indices of same observer F. G.	Variations in indices of different observers. F. G. & W.		Variations in indices of same observer W.	Opsonic mixture put up and indices calculated by W. — Index	Capsules of serum from which indices calculated Control	Patient
4.2	C.R.1. C.R.2	C.W.1 C.W.2	1·56 1·06	0·50	0·70 0·20	0·45 0·95	0·25	0·86 0·61	C.W.1. C.W.2	C.R.1 C.R.2
4.2	C.R.1 C.R.2	C.F.G.1 C.F.G.2	1·47 0·95	0·52	0·06 0·46	0·02 0·54	0·48	1·41 0·93	C.F.G.1 C.F.G.2	C.R.1 C.R.2
6.2	C.R.1 C.R.2	C.W.1 C.W.2	0·75 1·35	0·60	0·10 0·38	0·50 0·22	0·28	0·85 1·13	C·W.1 C.W.2	C.R.1 C.R.2
6.2	C.R.1 C.R.2	C.F.G.1 C.F.G.2	1·09 1·60	0·51	0·62 0·11	0·09 0·42	0·53	1·71 1·18	C.F.G.1 C.F.G.2	C.R.1 C.R.2
8.2	C.R.1 C.R.2	C.W.1 C.W.2	1·46 0·89	0·57	0·47 0·10	0·72 0·15	0·25	0·99 0·74	C.W.1 C.W.2	C.R.1 C.R.2
8.2	C.R.1 C.R.2	C.F.G.1 C.F.G.2	0·90 1·06	0·16	0·60 0·17	0·47 0·33	0·80	1·53 0·73	C.F.G.1 C.F.G.2	C.R.1 C.R.2
9.2	C.R.1 C.R.2	C.W.1 C.W.2	0·49 0·95	0·46	0·33 0·38	0·13 0·08	0·05	0·82 0·87	C.W.1 C.W.2	C.R.1 C.R.2
12.2	C.R.1 C.R.2	C.W.1 C.W.2	0·72 1·61	0·89	0·25 1·14	0·16 1·05	0·09	0·47 0·56	C.W.1 C.W.2	C.R.1 C.R.2
12·2	C.R.1 C.R.2	C.F.G.1 C.F.G.2	0·83 1·21	0·38	0·21 0·17	0·31 0·69	0·52	1·04 5·52	C.F.G.1 C.F.G.2	C.R.1 C.R.2
		Total	...	4·59	12·83		3·25	...		

Summary

Average difference between nine duplicate estimations by the same observer F.G. = 0·51, by the same observer W. = 0·36, and between 36 duplicate estimations by the two different observers = 0·36.

These writers also give an exactly similar series of indices, calculated from capsules C.W.1 and C.R.2 and C.W.2 and C.R.1, instead of from capsules C.W.1 and C.R.1 and C.W.2 and C.R.2.

We find in this series the figures are, nine duplicate estimations by observer F.G. = 0·55, by observer W. = 0·40, and 36 duplicate estimations by the two observers = 0·45.

This table has been analysed at length for three reasons:—

1. It gives the figures which can be most satisfactorily compared with ours.

2. The figures compared are the *indices*, i.e., the results of the method, which is a more practical test than statistics of the percentage differences between the highest and lowest counts obtained from various slides, etc.

3. As the indices compared are calculated from duplicate phagocytic mixtures the errors due to 'putting up,' as well as counting, are included, which is again a more practical test of the technique than a comparison of indices obtained from two observers counting the *same* slides.

The table shows that the average difference between duplicate indices calculated by F.W. and S. is more than twice as great as our own and more than four times as great as that of the workers quoted by Fleming. This inaccuracy may be partly ascribed to inexperience in technique, the enumeration of only fifty cells and employing too weak an emulsion, points which will be referred to later.

Other tables, II, III, XII, give the phagocytic counts obtained for two different capsules of the same blood, and their percentage differences, and also the percentage difference between the highest and the lowest phagocytic counts obtained each day with successive sets of fifty or one hundred cells. Thus on February 4th, 1907, F.G. found two counts from duplicate phagocytic mixtures were 65 and 36 respectively, the difference thus being 29. Now there are three possible ways of calculating the percentage difference of these figures:—

I. By placing the lower figure 36 in the denominator.

$$\frac{29 \times 100}{36} = 80 \cdot 6$$

II. By placing the higher figure 65 in the denominator.

$$\frac{29 \times 100}{65} = 44 \cdot 6$$

III. Placing the mean of the two figures (50·5) in the denominator.

$$\frac{29 \times 100}{50 \cdot 5} = 57 \cdot 4$$

It is clear that in I the error is largest and in II smallest.

Fitzgerald, Whiteman and Strangeways frequently calculate the percentage differences between the figures by placing the lower figure in the denominator, thereby making the error as great as possible. But they have no right whatever to assume that the lower figure is more correct than the higher. As it is impossible to determine which figure is more correct, the only reasonable method would have been to place the mean of the two figures in the denominator (Vide III). Fitzgerald, Whiteman and Strangeways by adopting Method I have made their errors appear as high as possible.

In Tables II and III, Fitzgerald, Whiteman and Strangeways show 'the phagocytic counts obtained for two different capsules of the same blood and their percentage difference.' For example, on 4th February, 1907, the counts obtained from two different capsules of the same serum (fifty cells counted) were:--

Capsule I.—36.
Capsule II.—65.

Now these writers estimate the percentage difference between these two counts as 80·6, in the manner we have described on page 310, but the percentage difference from the arithmetic mean would be, however, 28·7. As all our percentages have been calculated by the latter method, we have re-calculated the figures given by Fitzgerald, Whiteman and Strangeways in their Tables II and III, and can therefore compare them directly with our own.

TABLE IV, SHOWING PERCENTAGE DEVIATION FROM THE ARITHMETIC MEAN OF TWO PHAGOCYTIC COUNTS OBTAINED FROM TWO *Different* CAPSULES OF THE *Same* SERUM

Observer	No. of duplicate estimations	Organism	No. of cells counted on each slide	Average percentage deviation from the arithmetic mean
F. G.	22	Tubercle	50	14·3
W.	51	"	50	14·9
E. G.	30	"	120	5·3
E. G.	79	Staphylococcus	120	7·3

On account of the 'inconsistent results' obtained by Fitzgerald, Whiteman and Strangeways in their own experiments, a new series of observations was made by them upon eight slides, one from a normal and seven from tuberculous persons, prepared in another laboratory, the reputation of which should guarantee the opsonic technique being

'unquestionable.' From these slides several thousand leucocytes were counted in sets of twenty-five by one observer, F.G. The normal slide was unknown. In Table XII they give the percentage difference between the highest and lowest phagocytic count obtained on each slide in consecutive sets of 25, 50, 100 and 500 cells, and found if averaged for single cells to be 128·77, 70·82, 34·6 4·24, respectively, the maximum difference in the figures for 500 cells being 9·9 and the minimum 0·1. They state that this table proves clearly that 'the percentage difference on the results decreases enormously the greater the number of the cells that are counted.'

If the observers had adopted the usual method of calculating percentage differences these figures would have been much more favourable. However, taking them as they stand, it is clear that if 500 cells are counted their technique will give fairly accurate results, though, of course, no account is taken here of errors due to putting up the phagocytic mixtures.

Analysing further the counts of these eight slides, the observers show that the more cells counted the more the figures for the normal and tubercular sera tend to approximate. When the index is based upon 1,000 cells for the normal and control sera, the seven indices are 0·78, 1·04, 0·88, 0·86, 0·109, 0·98, 0·95 (Table XI, p. 136). They point out, further, that 'if only fifty cells are counted this might in most cases be sufficient to account for the differences recorded between normal and tubercular blood.' The suggestion obviously is that if sufficient cells are counted Wright's positive and negative phase would cease to exist. There is considerable force in this objection. But two facts must be remembered: firstly, that the normal limit for tubercular indices is probably, as Fleming has pointed out, 0·9 and 1·1, not 0·8 and 1·2. Second, it is quite possible that either the control serum was not normal or that the tubercular sera were drawn at a time when the indices happened to be nearly within the normal limits.

The occurrence, however, of a positive or negative phase has been noted by numerous independent observers again and again, and cannot be disproved by the examination of eight slides, even if they came from a laboratory above suspicion, and a thousand cells were counted from each.

Greenwood[1] has recently published a paper entitled 'A Statistical View of the Opsonic Index.' His conclusions, which are based upon Strangeway's counts of these eight slides and six slides counted in Wright's Laboratory, are the following:—

1. *Proceedings Royal Soc. Medicine*, Vol. II, No. 5, p. 154.

(1) Phagocytic distributions are markedly asymmetrical.

(2) This asymmetry, although reduced, is not removed by emulsions of (from the experimental standpoint) maximal thickness.

(3) The mode of a phagocytic distribution is a more reliable constant than the mean.

(4) A corollary of (1)—Positive and negative deviations will not occur in random sampling equally often.

Another set of figures which may compare with the results of Fleming and ours are those of Lloyd Smith, Radcliffe and Crossley.[1] (1) Here thirty-two duplicate indices for tubercle were estimated by two observers counting the *same* slides. Analysing the figures obtained from their charts I and II, we find the average difference between the duplicate indices is 0·26, the maxima being 1·1, 0·6 and 0·4. To the table we have also added some figures by French.[2]

TABLE V, SHOWING AVERAGE DIFFERENCE IN INDICES OBTAINED BY DIFFERENT OBSERVERS RECOUNTING THE SAME SLIDES

Paper by	Observer	Average difference	Number of pairs of indices compared	Approximate number of cells counted per slide	Approximate number of bacteria per cell	Organism
Fleming	2	0·064	41	100	2	Tubercle
Smith*	4	0·26	32	?	1·2	,,
French†	3	0·09	6	50	3	,,
,,	3	1·03	3	50	1·6	,,

*. *Lancet*, July 18, 1908.

†. *Practitioner*, 1906, p. 70.

‡. This is from an example, purposely given, of a very bad result.

Dr. Hort[3] sent capsules of the same blood drawn from tubercular or non-tubercular persons to 'experts' in 'well-known laboratories'; frequently duplicate capsules of a particular blood were sent to the same expert 'unknown to him.' Hort gives the result of three duplicate observations on three sera by the same observer T., the average difference being 0·10; and a similar number by the same observer O. and the same observer B., with an average difference of 0·12 and 0·10 respectively.

The figures obtained from examining the same serum in different laboratories were much more divergent, especially in the case of expert A., whose indices were usually far too high. For example, Test 3, observer

1. *Lancet*, July 18, 1908.
2. *Practitioner*, p. 70, 1906.
3. B. M. J., February 13, 1909.

A., tuberculo-opsonic index, 1·34: observer T., 0·67; observer T., 0·55. Test 6, tuberculo-opsonic index, observer A., 2·20; observer O., 0·96; observer O., 0·82; observer B., 0·82.

A. made no duplicate observations. The average error of T., B., and O. is slightly less than our own, though their figures are based on six indices instead of 120. It is noteworthy, however, that their emulsion yielded about 2·3 bacilli per cell, but that of expert A. only 1·1.[1]

Some Sources of Error

Assuming that the technique is carefully performed and none of the fallacies recently described by Fleming, such as agglutination of the erythrocytes, are introduced, two sources of error require special attention—the emulsion and the counting.

The Emulsion

Quality of the Emulsion.—We were surprised to find that the counts of tubercle bacilli were rather more accurate than those of staphylococci, in spite of the fragmentation, beading, and comparatively large and frequent clumps obtaining in the former organism and the small size of the clumps, rarely more than three cocci, in the latter.

Quantity of Bacilli in the Emulsion.—The quantity of bacilli in the emulsion is perhaps even more important. The figures published by Fleming show that the Wright School employed in 1907 a tubercle emulsion yielding about two, and a staphylococcus emulsion about three bacteria per cell in normal serum.

In Tables VI and VII we summarise the average errors in the four sets of triple indices estimated on the different days with different bacterial emulsions. The errors are grouped according to the strength of the emulsion employed each day. Thus, twelve indices with staphylococcus were estimated on July 23rd, May 28th, and August 1st, respectively, and on each of these three days the average number of staphylococci per leucocyte was less than 1·9. The average, however, for the three sets combined is 1·4 staphylococci per cell. The average error for this strength of emulsion, as calculated by *Methods A*, *B*, and *C* is 6·5, 0·25, etc. (See first line of table.)

1. Reyn and Kjer-Petersen of Copenhagen (*Lancet*, 1908, March 28, p. 919) in a most valuable paper criticise Wright's theory and technique rather severely, but we are unable to compare any of their results with ours.

TABLE VI. ILLUSTRATING THE RELATIONSHIP BETWEEN ERROR AND STRENGTH OF EMULSION

I—STAPHYLOCOCCUS ALBUS

Emulsion No. of bacteria per cell: Limits of groups	Average count per cell	Slides Error—Method A Average percentage deviation from the arithmetic mean of duplicate slides 60 cell counts	Indices Error—Method B Average difference between triple indices taken in pairs (i) on 120 cell counts	(ii) on 60 cell counts	Error—Method C Average maximum deviation from arithmetic mean of triple indices on 120 cell counts	No. of indices upon which preceding figures are based
Below 1·9	1·4	6·5	0·25	0·32	0·13	36
Between 2 and 2·9	2·2	5·1	0·17	0·19	0·13	60
Between 3 and 3·9	3·4	3·7	0·17	0·20	0·14	60
Between 4 and 4·9	3·7	4·5	0·14	0·22	0·13	24
Between 5 and 5·9	5·1	3·5	0·13	0·16	0·11	12
Above 6	6·3	4·0	0·11	0·15	0·10	48
Average	3·5	4·8	0·17	0·21	0·12	240

II—TUBERCLE

Emulsion No. of bacteria per cell: Limits of groups	Average count per cell	Slides Error—Method A	Error—Method B (i) on 120 cell counts	(ii) on 60 cell counts	Error—Method C on 120 cell counts	No. of indices
Below 1·9	1·7	2·5	0·22	0·22	0·16	12
Between 2 and 2·9	2·5	6·7	0·16	0·24	0·14	48
Between 3 and 3·9	3·3	6·2	0·16	0·15	0·13	24
Above 4	4·6	5·7	0·09	0·13	0·08	36
Average	3·2	5·9	0·15	0·19	0·12	120

VII—SUMMARY OF PRECEDING TABLE

I—STAPHYLOCOCCUS ALBUS

Emulsion No. of bacteria per cell: Limits of group	Average count per cell	Slides Error—Method A Average percentage deviation from the arithmetic mean of duplicate slides (60 cell counts)	Indices Error—Method B Average difference between triple indices taken in pairs (i) on 120 cell counts	(ii) on 60 cell counts	Percentage diminution in error on 120 cell counts	Error—Method C Average maximum deviation from arithmetic mean of triple indices (on 120 cell counts)	No. of indices upon which preceding figures are based
Between 1 and 2·9	1·9	5·6	0·20	0·24	16·6	0·13	96
Between 3 and 4·9	3·9	4·1	0·16	0·21	23·8	0·13	84
Between 5 and 6·9	6·0	3·9	0·12	0·15	20·0	0·10	60
Average	3·5	4·8	0·17	0·21	0·19	0·12	240

II—TUBERCLE

Emulsion No. of bacteria per cell: Limits of group	Average count per cell	Slides Error—Method A	Error—Method B (i) on 120 cell counts	(ii) on 60 cell counts	Percentage diminution in error	Error—Method C	No. of indices
Between 1 and 2·9	2·3	5·9	0·18	0·23	21·7	0·15	60
Between 3 and 4·9	4·1	5·9	0·12	0·14	14·3	0·10	60
Average	3·0	5·9	0·15	0·19	21·0	0·12	120

These tables demonstrate that there is a most definite connection between the error and the strength of the bacterial emulsion. This is especially obvious when large numbers of indices are grouped together, and when the error is calculated by *Method B*, which is the most practical and useful of the three methods. Naturally there are discrepancies, particularly in Table VI, but when it is remembered that we are dealing with two separate organisms, that the homogeneity of the emulsion, the quality of the film, and the accuracy with which they were counted by the observer (E. E. G.) necessarily varied somewhat from day to day, and, lastly, that the methods of calculating errors are somewhat rough, the fact that so few discrepancies occur is all the more striking.

The following deductions may be drawn from Table VII, in which the indices are arranged in the largest groups.

1. The error steadily diminishes as the strength of the bacterial emulsion increases as shown especially by Method B.
2. An emulsion of tubercle bacilli yielding about two or four bacilli respectively per cell, gives approximately as accurate results, as an emulsion of staphylococci yielding four or six cocci per cell. That is to say, the emulsions of staphylococcus should be somewhat stronger than those of tubercle. This also has been recommended by the Wright School.

We give below the percentage diminution in the error by increasing the strength of the emulsion.

Tubercle emulsion	increased	from	2·3 to 4·1	per cell, diminishes	error from	0·18 to 0·12 = 33 %
Staphylococcus	„	„	1·9 to 3·9	„	„	0·20 to 0·16 = 20 %
„	„	„	3·9 to 6	„	„	0·16 to 0·12 = 25 %

These figures indicate, as far as our work is concerned, that increasing the concentration of a tubercle or staphylococcus emulsion from two to four bacteria per cell diminishes the error by about 25 per cent.

The inaccuracy of Fitzgerald, Whiteman and Strangeways' work must partly be ascribed to the employment of an emulsion averaging only, as a rule, one to two bacilli per cell.

Lloyd Smith and his co-workers also employed a very weak emulsion, averaging, if one may judge from page 148, about one tubercle bacillus per cell in the control.

Allusion has already been made to the fact that in Hort's figures observer A., who obtained the most divergent results, employed the weakest emulsion.

The importance of a strong emulsion is confirmed by an analysis of

Fleming's figures, in which two observers, A and B, calculated forty-one duplicate indices by counting the *same* slides. Separating the indices into two groups, viz., those calculated from counts giving more than two bacilli per cell and less than two bacilli per cell, we find that in twenty-seven pairs of indices the average error was 0·07, and the strength of emulsion 1·6 bacilli per cell, and in fourteen pairs the average error was 0·05, but the strength of emulsion 2·4 bacilli per cell.

In our thirty-six observations (Table VI) with emulsions averaging 4·6 tubercle bacilli per cell, i.e., about twice the strength used in Wright's laboratory, gave an average error of 0·09, i.e., almost equal to Fleming's error of 0·07 for indices from duplicate phagocytic mixtures. This fact indicates that one of the main factors in the superiority of Fleming's workers lies in the greater uniformity of their emulsion, prepared, no doubt, from a strain of tubercle bacilli specially selected after continuous trial.

Probably the less homogeneous the emulsion the stronger it must be. There must be, however, an optimum strength for every emulsion above or below which the accuracy of the counts diminish. This optimum strength will depend not only on the nature of the organism and the tendency to clump, but possibly on the personal equation of the observer.

All the facts, then, favour strong emulsion and support the conclusions of Greenwood,[1] who, arguing from statistical considerations alone, stated 'that it is better to work with tolerably thick emulsion giving an average for normal serum of not less than three bacilli per cell.'

Owing to the laborious nature of the research from which the figures are obtained, most of the counting was done after the completion of the technique, so that, unfortunately, we did not realise the great importance of employing strong emulsion till too late. It must be remembered, however, that a high phagocytic count per cell demands considerably more time than a low one.

Counting

Method of Counting

Fleming holds that it is a 'great mistake' to count an 'arbitrary' number of leucocytes, for the observer should display some 'intelligence,' and the number counted should depend on the regularity of the count. This system is not a fair test of the accuracy of the opsonic technique unless the observer, having decided upon the number of leucocytes which give a true estimation of the phagocytosis of each slide, resolutely adheres

1. *Practitioner*, May, 1908, p. 645.

to his decision, even though he find the indices eventually calculated are contrary to expectation, or differ considerably from those of another observer. If such should happen, an enthusiastic and optimistic worker is exposed to the insidious temptation of either recounting the suspected slide or counting an additional number of cells in the hope that all may come right.

Perhaps our error might have been less had we counted in the manner suggested by Fleming, but then we might have unconsciously improved our results.[1]

Fitzgerald, Whiteman and Strangeways appear to have counted any polymorpho-nuclear cells indiscriminately on any part of the slide. Moss noted that the highest counts occur towards the end of the slide, and particularly is this evident if larger groups are considered. He attributed this result to a sorting out process when making the smear, whereby the largest cells tend to be left to the end.

As previously stated, we made it a rule to count thirty cells on each of the two borders of a film from the end of the smear, where the leucocytes are most abundant, passing backwards towards the beginning. Analysing the results of this method, it appears in a consecutive series of 311 slides that the number of bacteria in the first ten of the thirty cells averages 2·7 per cent. less than in the last ten cells counted.

In another series of 100 slides the figures were 2·6 per cent. less for tubercle and 1·9 per cent. less for staphylococcus.

These results contradict Moss's statement, but he divided his slides into zones 1 cm. wide and counted 150 cells in each zone. Our figures, however, suggest the advisability of counting as far as possible corresponding portions of every slide. We believe that leucocytes should never be selected from the middle zone of the smear (*AA* in the diagram), for here, the film being thicker, the leucocytes tend to be contracted, so that their cytoplasm stains more intensely, and the contained bacteria are more difficult to count.

Of course all damaged leucocytes in which the outline is indistinct, owing to damaged cytoplasm, must be rigorously excluded from the counts.

Number of Cells Counted

We have already drawn attention to the fact that the technique of Fitzgerald, Whiteman and Strangeways, as tested by their own figures in Table II, is much less accurate than our own, but they enumerated the bacteria in only fifty cells, selected apparently indiscriminately, and

1. See footnote, p. 321.

employed an emulsion yielding about two bacteria per cell, while we counted 120 cells, selected with method, and the emulsion averaged 3·2 bacteria per cell.

In order to equalise the conditions as far as possible, we have re-calculated our indices from figures obtained by counting the first sixty cells only, instead of the whole 120. (See Tables VI, VII.)

These tables demonstrate that even with a weak emulsion yielding on the average 2·3 bacteria per cell, our average error is 0·23 for tubercle as compared with their figures of 0·51 and 0·55, 0·36 and 0·40, 0·36 and 0·45. Even after making due allowances for the slight advantage to us of enumerating sixty instead of fifty cells, of using some phagocytic mixtures in duplicate (see p. 302), and from the somewhat stronger emulsion, it is clear that our work is decidedly more accurate than theirs, as it is less accurate than that of Fleming.

It will be noticed that on one occasion the error appears less on the first sixty than on the total 120 for tubercle. This is an accident, and would be more than neutralised by the counts of the second sixty cells; thus, on July 20th, the average error for staphylococcus was 0·09 with 120 cells, 0·05 with the first sixty, but 0·135 with the second sixty.

The following conclusions may be drawn from perusal of Table VII:—

1. The error diminishes by doubling the number of cells counted in the case of both tubercle and staphylococcus by some 20 per cent., but not by a half. This agrees with Greenwood's comment—'the belief that a sample of fifty cells is twice as good as one of twenty-five indicates a somewhat primitive state of knowledge.'

2. Sixty cells with an emulsion of four bacteria per cell gives approximately as accurate results as 120 cells with an emulsion yielding about two bacteria per cell, in the case of both tubercle and staphylococcus. (See Chart, p. 320.)

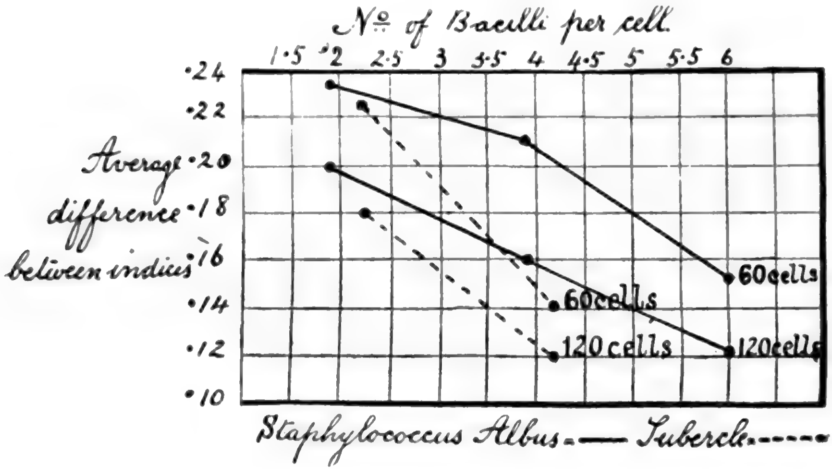
Fitzgerald, Whiteman and Strangeways speak of the absolute necessity of 'enumerating at least 1,000 cells.' But 'even' by so doing a percentage difference of 25 might occur. If this statement were invariably true, then Wright's technique is practically useless, not only for the estimation of opsonins, but also for any experimental work in which degrees of phagocytosis are compared.

The figures given below, however, should convince the most hostile critics that Wright's technique measures something! They represent four sets of indices, each estimated nine consecutive times on the same day, August 10th, 1908. These indices are calculated from twenty-seven

separate phagocytic mixtures, nine being used as controls in series I and II, and a second nine in Series III and IV. 120 cells were counted, and there were no duplicate estimations.

'A'		'L'	
I	III	II	IV
Opsonic indices	Cytophagic indices	Opsonic indices	Cytophagic indices
1·34	0·90	1·36	0·98
1·08	0·79	1·20	0·86
1·05	0·78	1·13	0·85
1·04	0·76	1·08	0·85
1·02	0·73	1·05	0·84
1·00	0·73	1·03	0·83
1·00	0·73	1·03	0·77
0·96	0·62	0·93	0·73
0·86	0·58	0·88	0·63
Average 1·04	0·73	1·08	0·81

If we have exhibited some of the work of Fitzgerald, Whiteman and Strangeways in an unfavourable light, it is because we believe that we have discovered the main reasons for their imperfect technique. They deserve the best thanks of everyone interested in opsonic work, for the courageous publication of their results.



SUMMARY AND CONCLUSIONS

1. The accuracy of the opsonic technique of Wright and Douglas has been vigorously impugned by many writers, notably Simon, Bolduan, Strangeways and Greenwood.

2. In 1907 Wright stated that the error in estimating the tubercular opsonic indices for normal bloods is rarely more than plus or minus 5 per cent.

3. Next year Fleming pointed out that duplicate estimations of tuberculo-opsonic indices usually differ from each other by less than 20 per cent., provided the observer counts 'intelligently.'

4. We have estimated a large number of consecutive indices with tubercle and staphylococcus three times, and find that the average difference between each set of triple indices taken in pairs is 0·15 with tubercle and 0·17 with staphylococcus. These figures may be compared with 0·07 obtained by Fleming[1] and 0·51 and 0·55, 0·36 and 0·40, 0·36 and 0·45 obtained by Fitzgerald, Whiteman and Strangeways in a series of duplicate estimations with tubercle.

5. We adopted all the precautions recently advised by Fleming in our work. The possibility of auto-suggestion influencing our counts was eliminated.

6. The accuracy of our technique was closely dependent upon two variable factors:—(*a*) The number of cells counted; (*b*) the strength of the bacterial emulsion.

7. The error steadily diminishes as the strength of the bacterial emulsion increases. By increasing the concentration of a tubercle or staphylococcus emulsion from two to four bacteria per cell the error diminishes about 25 per cent.

8. An emulsion of tubercle bacilli yielding about two or four bacilli respectively per cell gives approximately as accurate results as an emulsion of staphylococcus yielding four or six cocci per cell.

9. The error diminishes by doubling the number of cells counted from 60 to 120 in the case of tubercle and staphylococcus by some 20 per cent.

1. Greenwood concludes that 'Dr. Fleming's counts show signs of not being random samples but selections.' We are not clear whether this criticism applies to these figures from the *Practitioner*. (Greenwood, *Proc. Roy. Soc. Medicine*, Vol. II, No. 5, p. 151.)

10. Sixty cells with an emulsion of four bacteria per cell give approximately as accurate results as 120 cells with an emulsion of about two bacteria per cell, in the case of both tubercle and staphylococcus.

11. Assuming the truth of Wright's dictum that the opsonic index is an index of the 'power of phagocytic response' (although we claim to have demonstrated elsewhere that this dictum is not true), the inaccuracy of the opsonic technique is such that at present we attach no importance to an index between 0·8 or 1·2 (even estimated by an expert) in the diagnosis and treatment of disease, unless the observation had been repeatedly confirmed.

12. We entirely disagree with those critcs who appear to maintain that the technique of Wright and Douglas is practically useless as a means of comparing degrees of phagocytosis.

THE RELATIONSHIP OF DOSAGE OF A DRUG TO THE SIZE OF THE ANIMAL TREATED, ESPECIALLY IN REGARD TO THE CAUSE OF THE FAILURES TO CURE TRYPANOSOMIASIS, AND OTHER PROTOZOAN DISEASES IN MAN AND IN LARGE ANIMALS

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(*Received June 11th, 1909*)

It is an almost universal custom at the present time in describing scientific work of a pharmacological or therapeutic nature intended either to establish a lethal or a curative dose, to state the dose as so much per kilogram of body-weight of animal or man employed as the subject of experiment or treatment.

Many of the observers who use this method of expressing results are aware and state that it is only roughly accurate, and that experiments must be made from species of animal to species of animal on account of idiosyncrasies.

The object of this note is to point out, that, quite apart from idiosyncrasies, and alteration in the species of animal, this method of stating dosage in reference to body-weight is not only inaccurate, but rests entirely on a wrong principle for many kinds of drugs, which, even in the same animal species, act upon two individuals of different size, not proportionately to their weights, but proportionately instead *to their body-surfaces* or, in other words, proportionately to the two-thirds powers of their weights, which leads to quite different doses.

For example, the dose of many drugs which can be given to children or infants to produce a given therapeutic result, is often many times larger than the proportionate dose for an adult, on the basis of body-weights. There are a few cases such as the preparations of morphia where the child has a marked idiosyncrasy or sensitiveness to the drug, but in the majority of cases the balance is entirely in the other direction, and an adult of say 150 pounds weight cannot be given 15 times the dose of an infant of 10 pounds, but much more nearly a dose of 6 times as much, which is the two-thirds power of the ratio in the two weights.

Not only is this relationship of importance in regard to the method of expressing dosage and determining the approximate dose in man or large animals, from experiments made upon smaller animals. It is also

of the utmost importance in that it naturally sets a limit to our power of applying therapeutic agents against disease in larger animals, and allows a cure with ease in smaller animals, which is difficult or impossible in man or large animals, simply because they are large and not because of any particular sensitiveness to the drug.

For example, it is perfectly easy to cure trypanosomiases by atoxyl or other organic arsenical preparations, or better still by a proper combination of arsenic and mercury, in small animals such as the mouse or rat. The difficulty increases with rabbits, but in a large number may still be surmounted, as also in small monkeys. But with donkeys, cattle, horses and men, the difficulty is enormously increased, the trypanosomes can scarcely be driven out from the circulation by such sub-lethal doses as it is possible to give, recurrence sooner or later takes place and the animal or man succumbs. This is the common experience of all workers in the fight against the trypanosome group of diseases, and is particularly well seen in the work of Moore, Nierenstein and Todd upon treatment by atoxyl followed by mercury. Like other workers we were in all cases able at once to drive the parasites out of the peripheral circulation *in rats* with atoxyl and by then giving mercury were able to keep them permanently from recurring in a high percentage of cases (about 60 per cent.).

But when we came to test this greatly improved result as seen in rats to the larger mammalia, our results took on quite another complexion, and of 15 donkeys we were unable ultimately to save one. In the first case, on account of the limitations in the relative dose we could not satisfactorily and at once with a single dose drive the parasites out from the peripheral circulation. In some of our experiments for considerable intervals no parasites could be found even by most careful examination, but since in other donkeys similarly treated, a small number of parasites kept persistently present and finally became inured or 'Fest' to our drugs, we could not be free from suspicion, that the parasites *in very small number* so as to escape actual observation, were always present, and that our practically complete failure with the large animals, using a method of treatment almost perfect for the rat, was due chiefly to this cause. That is to say, in the large animal the dose cannot be given proportionately to the body-weight and so the trypanosomes cannot all be killed.

Exactly the same results follow for the admirable treatment by antimony, introduced first by Plimmer and Thomson, good results follow and often a high percentage of absence of recurrence in rats and small animals,[1] but in larger animals and man the percentage is much lower, although some cases of cure have been noted.

1. I can completely confirm this result from a large number of experiments made in collaboration with Dr. T. T. Bark in this laboratory. We found that even smaller doses than recommended by these authors drove the parasites out. Also a careful search of all the heavy metals, elements of the phosphorus group, and rare elements, gave negative results, except in the cases of antimony and arsenic only.

A like result has been obtained with other organo-arsenical compounds.

In all these cases it is to be observed (with the possible exception of the dog[1]) that the difficulty is not one of idiosyncrasy either to parasite or drug in a particular animal species, but that definitely in each case, and from each group of observers, we have the uniform report that small animals are easily treated and large animals including man are difficult of treatment.

It is hence of peculiar interest to review the situation from the general point of view, and try to find whether there is any physico-chemical basis which may supply an explanation. This is particularly so, since a side-light is cast upon the general bio-chemical question of what regulates the maximum therapeutic dose possible with such drugs in these diseases due to protozoa or other micro-organisms.

The establishment of the minimum lethal dose of atoxyl proves at once that this dose is not proportional to the body-weight, but relatively falls off very rapidly as the body-weight is increased.

Thus, a large sized rat of say 140 grams weight can safely be given about 0·4 c.c. of a 5 per cent. solution of atoxyl, that is about 0·02 gram. Now, if the dose could be given proportionately to body-weight, a man of 70 kilograms weight, since he weighs 70,000 grams or 500 times as much as the rat, ought to be able to safely stand 500 times as much atoxyl, that is $0{\cdot}02 \times 500 = 10$ grams or in 5 per cent. solution $= 200$ c.c.

As a matter of fact, he not only cannot stand approximately this quantity, he can only stand a small fraction of it, the highest dose which can be given being one gram or one-tenth of the above amount calculated from relative body-weight, and this is not an idiosyncrasy of man, but is true for all large animals.

If now instead of body-weights, the two-third power of the body-weights be taken for the purpose of estimating the relative doses. We have that the ratio of weights is 1 : 500 and the two-thirds powers of this ratio gives the ratio approximately of 1 : 63, and if 0·02 grams is the dose for the rat, on this basis the dose for the man will be $0{\cdot}02 \times 63 = 1{\cdot}26$ grams.

Now this very closely represents the amount found by actual experimentation, as 1 gram of atoxyl has frequently been given as a therapeutic dose in man.

When the cause of this relationship is further investigated, it is found

1. It may also be noted that the dog has a very small area of intestine relatively to its body-weight.

to lie in the selective action of arsenical and antimonial compounds upon cells lying upon certain surfaces of the body.

These substances are selectively taken up by epithelial surfaces.

It is well known that arsenic and antimony and indeed most of the heavy metals such as zinc, silver and copper, when taken by the mouth act as surface irritants upon stomach and intestine. It is less well known and only for certain heavy metals, but is none the less a general law, as experiments have shown me, that all the heavy metals are selectively taken up by the cells lining the intestine, and kill by extensive inflammation of the intestinal mucosa. Thus I have found that after *subcutaneous* injection, of soluble salts of lead, tin, bismuth, iron, copper, nickel, cobalt, or antimony, there is extensive inflammation of the gastro-intestinal tract, often accompanied by multiple hemorrhage and ulceration.

The same inflammatory processes in the intestine are found after all forms of arsenic including atoxyl.

This shows that the intestinal mucous membrane acts as the excretory organ for these foreign substances, and hence takes them up selectively from tne circulation.

Accordingly the minimal lethal dose or the full therapeutic dose will be limited by the concentration to which that dose gives rise in the epithelium cells of the intestine.

Now, placing on one side variations in length of intestine, and variations in length of intestinal villi, this means that the maximum dose is proportional to the area of intestine which the animal possesses.

Hence, if for simplicity we suppose that the animal from small size grows symmetrically bigger in all proportions so as to form a bigger animal which is a *fac simile* of the smaller, then we must inevitably have from purely geometrical considerations of the dimensions of solid figures, that when the length, or linear dimensions are say doubled, the surfaces external and internal of the body increase four-fold, and the cubic dimensions, whch are of course proportional to the body-weight, increase eight-fold.

Put otherwise this means that the dimensions of surfaces such as area of skin, lung area and intestinal area increase as the two-thirds power of the body-weights.

Now a great number of drugs including, as above mentioned, atoxyl and all salts of heavy metals attack cells spread out on a surface and hence the bigger animals cannot stand proportionately large doses, and, for example, an animal eight times as heavy as another cannot be given

eight times the dose, but only $8^{\frac{2}{3}}$, that is approximately four times as much.

But the result of this is that the concentration of the drug in the tissue fluids and blood of the heavier animal will only be approximately one-half of that in the smaller animal.

Suppose now that both animals have been affected by trypanosomes and we have been attempting to kill them in both, then it is obvious that there is far more margin to come and go on in the smaller animal than in the larger one

The trypanosome is swimming about in the blood plasma of the animal, it may be a rat in one case and a horse in the other, the factor it has to deal with is the concentration to which the drug can be raised in that plasma, it is indifferent what the host may be. But as regards the host, the drug is taken up by the intestinal cells selectively, and the amount of drug which can be given is not determined directly by the concentration in the plasma, but by the number of columnar intestinal cells, that is by the area of intestine which is proportionately greater in the smaller animal.

This fact that the surfaces are relatively larger in smaller animals than in larger animals, has been thoroughly appreciated in regard to body-temperature and its variations.

A small animal is more susceptible to changes of temperature than a large one, it requires more food proportionately to its weight than a larger animal, and for the same reason its temperature goes up and down more readily because the fly-wheel of weight is smaller relatively to the amount of surface through which heat exchanges are going on, that is the working part of the engine. It is also obvious since the surfaces are the working parts of the body, and these are relatively greater in proportion to the amount of material re-acting (*i.e.* body-weight) that all the processes are quickened up in the small animal, so that there is an exceedingly rapid heart-beat and respiration in all small mammalia.

In cold-blooded animals and invertebrates, a different chemical constitution in the protoplasm with a different reactivity and speed of oxidation sets a new limit.

Returning to the pharmacological aspect, we find that this same factor of relative surfaces and volumes is in operation, but has not hitherto been sufficiently recognised, and that it lies fundamentally at the bottom of the difficulty of treating man or large animals.

A careful consideration of the experimental facts known regarding the action of atoxyl and similar therapeutic agents upon trypanosomes has

led me to the belief, that the intestine not only acts as above pointed out in limiting the possible therapeutic dose, but also that the cells of the intestinal mucosa, are essential intermediaries in preparing or elaborating in a bio-chemical fashion from the atoxyl or other substance administered, a substance which is peculiarly deadly for the trypanosome.

This is shown by the fact so well known to all workers upon trypanosomiasis, that substances can be found out by the score which even in minimal concentration are deadly to trypanosomes in saline in a watch-glass, yet these substances can be subcutaneously injected into animals infected with trypanosomiasis without producing the slightest obvious effect upon the parasites.

The explanation here probably is that the said substances are thrown out of solution by the plasma proteins, which are not present to protect the parasites in the experiment with the trypanosomes in saline *in vitro.*

A still more remarkable and instructive experiment, well known also to workers on the subject, is that trypanosomes may be treated *in vitro* in saline, with concentrations of atoxyl, and other trypanocidal drugs, many times higher than the lethal concentration required to destroy them after subcutaneous injection in the animal, without producing the slightest apparent effect upon the parasites, which go on moving about vigorously for hours.

The inference is obvious, viz.: that there must be formed *intra vitam* by some bio-chemical process, an organic body especially lethal to trypanosomes.

Now it has been shown that the seat of bio-chemical activity after administration of arsenic or antimony is the intestinal mucosa, and it would seem probable that this is also the situation of manufacture of the trypanocidal virus.

If this be the case, it is clear that the larger animal is doubly handicapped, for in the first place the therapeutic dose is lowered because of the relatively smaller number of intestinal cells to take up the drug, and their consequent rapid poisoning and death of the animal if the attempt be made to give the relative dose; and secondly, that this relatively smaller number of cells or laboratories must first act upon the drug before it can be turned out as a trypanocide upon the parasites.

These reflections suggest certain lines of experimentation and treatment, which, since I am not myself in a position to experiment upon larger animals, I here outline for others who may desire to test them.

In the first place, an attempt might be made to prepare the trypanocidal material by giving gradually increasing doses of atoxyl in

healthy animals, and then using an extract of the intestinal mucosa in full doses as a therapeutic agent in man if it were found to be active in small animals. Such an extract of the intestinal mucosa ought to be active against trypanosomes in vitro.

Secondly, it appears to me that an attempt might be made to increase the production of anti-trypanocidal virus by giving atoxyl by mouth simultaneously with subcutaneous injection.

Lastly, as a kind of opposed treatment, the attempt might be made to increase the possible subcutaneous dose by giving an antidote by the mouth to the arsenic or antimony just before injection, so as to protect the intestinal cell at the first pressure of the drug in the circulation due to the subcutaneous injection.

Summary

1. In the case of substances which act by stimulation or inflammation of surfaces, such as the intestinal tract, the maximum dose is proportional not to the body-weight, but to the two-thirds power of the body-weight.

2. This leads to important differences in dosage in man and large animals.

3. It also shows that the possibilities of treatment are diminished by natural means in man and large animals. These animals have naturally less intestinal, and other, surfaces per unit of weight; accordingly they can only take up proportionately less drug, and if any remedial substance is manufactured by the surface cells, they can only manufacture relatively less of this than the smaller animal.

4. Also in general terms, uptake and output of poison or infection are relatively more rapid in the small animal. The small animal and child are hence at the same time more susceptible to onset of infection, and have more power of recuperation when infected.

Note. The practical suggestion may be made that for the great majority of drugs the method of stating dosage as so much per kilogram should be abandoned.

The following method is suggested:

The dose for an animal of an observed weight should be determined, then by taking the two-thirds powers of the two weights, the dose for an animal of, say, 1 kilogram, can readily be calculated. This should be

stated as the dose for a one kilogram animal, not as dose per kilogram. From this dose, the dose for an average-sized human being of, say, 64 kilograms, can now quite readily be deduced. It is the dose for the kilogram animal, not multiplied by 64, but by the two-thirds power of 64, which is 16. Here it is obvious that the usual method of calculating dose per kilogram would have given a dose no less than four times too large. It is interesting to note that this new rule shows why, in human therapeutics, the dose for low weight and high weight individuals does not fall and rise in direct proportionality to the weight, buf in a somewhat lessened ratio. The heavier person requires a little more, but not proportionately to increased weight, and *vice versa*.

PROPOSALS FOR THE NOMENCLATURE OF THE LIPOIDS*

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The term 'lipoid' was used fifty years ago by Kletzinski, but has only obtained importance since Overton (1901) re-introduced it in connection with H. Meyer's and his own theory of narcosis.[1] It is now generally accepted as a generic name for all those 'fat-like' constituents of animal or vegetable cells which can be extracted by means of ether or similar solvents. If we accept this definition which from its origin is mainly a biological one, we are forced to include amongst the lipoids substances which have very little in common from a chemical point of view.

The fundamental importance of these substances in biological processes is being more and more demonstrated by exact experimental investigations, especially in relation to problems of immunity. These investigations have drawn attention to the incompleteness of our chemical knowledge of these substances, and stimulated research on their chemical constitution, a thorough knowledge of which seems to be the first essential condition for the elucidation of the biological questions.

Unfortunately there still exists a great confusion in the nomenclature of these substances. This confusion is mainly due to three reasons: (1) Substances which are evidently identical have received different names from different observers; (2) the same name has been applied to different substances; (3) several names which have no chemical meaning are used in a general physiological and histological sense.

In order to arrive at a uniform nomenclature, it seems desirable (1) to omit, for the present, all those names which have been given to substances which are either insufficiently characterised, or the existence of which has not been verified by later researches; (2) to dismiss altogether the names given to substances which do not represent definite chemical compounds, and (3) to adopt, in the case of different names for the same substance, those names which were proposed by the original discoverer.

The following nomenclature is based on the classification introduced by Thudichum, the value of whose pioneer work in this field is now generally recognised, after it has been neglected for nearly thirty years.

* A paper read before the Physiological-Chemical Section of the Seventh International Congress of Applied Chemistry, May, 1909.

1. Overton, *Studien über die Narkose*, Jena, 1901.

This classification offers the advantage of easily allowing for extension and of having been already adopted in its main outlines by the majority of modern workers (Schulze, Winterstein, Hammarsten, Erlandsen, Bang, Thierfelder, Fränkel, etc.).

We may distinguish three large groups of lipoids:

(I) *The Cholesterin group* (free from both phosphorus and nitrogen).

(II) *The Cerebro-Galactosides* (free from phosphorus but containing nitrogen).

(III) *The Phosphatides* (containing both phosphorus and nitrogen).

(I) *The Cholesterin group.*[1] The main representative of this group is *cholesterin.* The name cholesterin has the advantage of long and international usage to recommend it in preference to 'cholesterol,' which latter name only represents one characteristic of the latter substance, namely, its alcohol nature. This group also includes the vegetable cholesterins, usually called *phytosterins.* Pigments such as *lipochromes,* as well as odoriferous substances which have not yet been chemically characterised may also be provisionally included in this group, for at any rate they resemble cholesterin in being free from both phosphorus and nitrogen.

(II) *The Cerebro-Galactosides* represent a group of nitrogenous phosphorus-free substances which are characterised by the fact that its members furnish galactose on hydrolysis. The general name of this group which was first used by Thudichum, is preferable to the shorter name 'Cerebrosides' since 'cerebrose' is identical with galactose. Two substances belong to this group, namely, *Phrenosin* and *Kerasin,* the latter of which has not yet been so well studied as the former.

In adopting the name *Phrenosin* a number of names must be discarded, which have given rise to a great deal of confusion. Phrenosin was recognised by Thudichum as the main phosphorus-free constituent of the mixture 'Protagon' (=Cérébrote of Couerbe, 1834; Cerebric acid, Fremy, 1841), and he proposed this name in preference to 'Cerebrin,' as the latter term had been applied to widely different substances.

The name 'cerebrin' was originally given by Kühn (1828) to a mixture of phosphatides and cholesterin; it was used by Gobley (1850) for a substance which, according to its preparation (boiling with sulphuric or hydrochloric acid), must have been a partially hydrolysed product, still containing phosphorus. Müller (1858) for the first time applied this

1. The general name, 'Sterin,' has been proposed for this group by Abderhalden. Apart from other objections, the possible confusion with 'Stearin,' from which the name is derived, will probably stand in the way of its general acceptation.

name to a phosphorus-free substance, obtained by a process in which brain had been coagulated by means of baryta or lead acetate. Müller's 'cerebrin' probably represented also a partially hydrolysed product and has not since been obtained. Some time after Thudichum described phrenosin, Gamgee obtained the same substance and called it provisionally 'Pseudo-cerebrin.' Parkus (1881) and Kossel and Freytag (1893) again used the name 'cerebrin' for a phosphorus-free substance similar to phrenosin, in the preparation of which, however, boiling baryta had been used. Finally Wörner and Thierfelder (1900) gave the name 'Cerebron' to a phosphorus-free substance, which they prepared mainly by fractional crystallisation from so-called 'protagon,' and which was found to be identical with Gamgee's 'pseudo-cerebrin.'

It seems certain that 'cerebron' and 'pseudo-cerebrin' are identical with phrenosin, and it is therefore desirable to retain the original name ***phrenosin***, and to dismiss the others.

Kerasin was first obtained by Thudichum. Parkus described a similar substance, obtained by his baryta process, which, however, he called 'Homocerebrin.' The name kerasin was again used by Kossel and Freytag for their preparation, which resembled that of Thudichum's. The substances called by Bethe 'amino-cerebric-acid glucoside' is also probably identical with kerasin.

(III) *The Phosphatides.* This name was proposed by Thudichum for a group of substances which contain both phosphorus and nitrogen. It was re-introduced by Schulze and by Hammarsten, and is now generally accepted, the name 'lecithans' proposed by Koch not being general enough. Thudichum demonstrated the existence of a whole series of these substances, where formerly the presence of only one, namely 'Lecithin,' has been assumed. He introduced the principle which guides modern workers in the classification of these substances. The phosphatides, according to this, may be divided into several sub-groups, according to the ratio of nitrogen to phosphorus contained in them. For the present the constancy of this ratio (after repeated re-crystallisations and fractionations) offers the only index for the chemical entity of these substances.

We may distinguish the following sub-groups:—

(1) Monoamino-monophosphatides .... N : P = 1 : 1
(2) Diamino-monophosphatides .... N : P = 2 : 1
(3) Triamino-monophosphatides .... N : P = 3 : 1
(4) Triamino-diphosphatides .... N : P = 3 : 2
(5) Monoamino-diphosphatides .... N : P = 1 : 2

It is obvious that this list may be easily extended as phosphatides possessing a different N : P ratio are isolated.

(1) *Monoamino-monophosphatides.* This sub-group comprises *Lecithin* and *Kephalin.* The term 'lecithin,' which was formerly given to a mixture of phosphatides, should be restricted to those monoamino-monophosphatides which are soluble in ether, and not precipitated from their solution by alcohol. The nature of the fatty acids contained in them is still uncertain, but oleic acid seems to be the characteristic one. Kephalin (or kephalins) first described by Thudichum, is soluble in ether, and precipitated from this solution by absolute alcohol. It probably contains as its characteristic fatty acid a still more unsaturated acid than oleic acid. (Zuelzer has given the name Myeline to a substance obtained from egg yolk, which is evidently similar to or identical with Thudichum's Kephalin).

Another substance belonging to this group has recently been isolated from ox pancreas by Fränkel and Pari. This monoamino-monophosphatide which has been called *Vesalthin,* furnishes on hydrolysis myristic acid, besides an unknown unsaturated fatty acid and a base different from choline.

Thudichum includes in this group another substance called by him Myeline. In view of the fact, however, that this substance has been very little studied, and in order to avoid confusion with the general term 'myelination' as used in the physiological sense, it seems desirable for the present to dismiss the name altogether.

(2) *Diamino-monophosphatides.* The main representative of this group is *Sphingomyelin.* This phosphatide, which, in distinction from the waxy lecithin and kephalin, is a crystalline white substance, was also first obtained by Thudichum. It represents the main phosphatide of the so-called 'protagon' mixture. Its occurrence in brain (and probably in the cortex of the adrenals) has been confirmed by Rosenheim and Tebb, who prepared it by a new method. A similar diamino-monophosphatide was obtained as a cadmium salt by Erlandsen from the heart muscle, and by Thierfelder and Stern from egg yolk.

(3) *Triamino-monophosphatides.* A phosphatide belonging to this group has recently been isolated by Fränkel and Bolaffia from egg yolk. They gave the name *Neottin* to this triamino-monophosphatide.

(4) *Triamino-diphosphatides.* A substance belonging to this class is described by Fränkel and Nogueira, who obtained it from ox kidney.

(5) *Monoamino-diphosphatides.* The first phosphatide of this group was discovered by Erlandsen in heart muscle, and called by him *Cuorin.*

Recently MacLean demonstrated the presence of another member of this group in egg yolk. Both substances are highly unsaturated and yield on hydrolysis a base different from choline.

In this classification the lipoids which contain sulphur, although possibly of considerable importance, have not yet been considered, as they have so far not been isolated in a sufficiently pure state to warrant their inclusion. Thudichum's classification provides for them in the second group as Cerebro-sulpho-galactosides, or if found to contain phosphorus as well, in the third group as amino-sulpho-phosphatides.

It is proposed to discard the following names:—

Cérébrote Cerebric acid Protagon	Mixtures of various lipoids.
Cerebrin Pseudo-cerebrin Cerebron	Identical with phrenosin.

Homocerebrin—identical with kerasin.
Myelin—for reasons explained above.

The following table contains a list of the substances which are included by the proposed classification.

I. *The Cholesterin group.*
   1. Cholesterin.
   2. Phytosterins.
   3. Lipochromes, etc.

II. *The Cerebro-Galactosides.*
   1. Phrenosin.
   2. Kerasin.

III. *The Phosphatides.*
   1. Monoamino-monophosphatides N : P = 1 : 1.
      a. Lecithin (or Lecithins).
      b. Kephalin (or Kephalins).
      c. Vesalthin.
   2. Diamino-monophosphatides N : P = 2 : 1.
      Sphingomyelin.
   3. Triamino-monophosphatides N : P = 3 : 1.
      Neottin.
   4. Triamino-diphosphatides N : P = 3 : 2
   5. Monoamino-diphosphatides N : P = 1 : 2.
      Cuorin.

In the present state of our knowledge of the lipoids it seems desirable to defer the introduction of new names until a complete study of the hydrolytic cleavage products makes it evident that the substance dealt with is fundamentally different from those previously described. The isolation of the lipoids (except cholesterin) in a chemically pure state is almost as impossible at present as that of the proteins and we are forced to rely, just as in the latter case, mainly on the results of complete hydrolysis for further information about these substances.

A COMPARISON OF THE METHODS FOR THE ESTIMATION OF TOTAL SULPHUR IN URINE

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It is well known that urine contains sulphur, not only in the form of sulphates, both inorganic and ethereal, but also in the form of less highly oxidised organic compounds, generally spoken of (following E. Salkowski's[1] suggestion) as 'neutral sulphur.' The organic compounds in question are very diverse and include thiocyanic acid and its salts,[2] cystine and closely related bodies,[3] taurine and tauro-carbamic acid,[4] methyl mercaptan,[5] ethyl sulphide,[6] thiosulphuric acid,[7] sulphurous acid,[8] urochrome,[9] oxyproteic acid,[10] uroproteic acid,[11] and uroferric acid.[12]

The quantitative determination of the total amount of sulphur present in the urine depends upon the fact that this neutral or unoxidised sulphur can be converted by the aid of suitable oxidising agents into sulphuric acid. The various methods in use differ mainly in the different oxidising agents chosen. They may be arranged in three groups:—

1. *Virchow's Archiv.*, Bd. LVIII, S. 472, 1873.

2. Leared, *Proc. Roy. Soc., Lond.*, Vol. XVI, p. 18, 1870; R. Gschleiden, *Tageblatt d. 47. Versammlung deut. Naturf. u. Aertzte in Breslau*, 1874 (quoted from E. Abderhalden's *Lehrbuch d. physiol. Chem.*, II Aufl., S. 343, 1909); I. Munk, *Virchow's Archiv.*, Bd. LXIX, S. 354.

3. Stadthagen, *Zeitsch. f. physiol. Chem.*, Bd. IX, S. 125, 1885; E. Goldmann u. E. Baumann, *ibid.*, Bd. XII, S. 254, 1888.

4. E. Salkowski, *loc. cit.*

5. M. Nencki, *Arch. f. exper. Path. u. Pharmak.*, Bd. XXVIII, S. 206, 1891.

6. J. J. Abel, *Zeitsch. f. physiol. Chem.*, Bd. XX, S. 253, 1894.

7. J. J. Abel, *loc. cit.*, for dog. Presch found in case of typhus that a compound which he isolated from the urine in small quantities gave sulphurous acid on distillation with acids. This is the only case I can find in which thiosulphuric acid has been noticed in urine of man. Quoted from A. Heffter, *Ergebnisse d. Physiol.* (Asher and Spiro), Jg. I, Abt. I, S. 458, 1902.

8. Strümpell, in case of fever, *Arch. d. Heilk.*, 1876. (Quoted from Dixon Mann, *Physiol. and Path. of Urine*, 2nd Edit., p. 18, 1908.)

9. St. Dombrowski, *Zeitsch. f. physiol. Chem.*, Bd. LIV, S. 204, 1907-8.

10. St. Bondzýnski u. R. Gottlieb, *Centralb. f. d. med. Wissensch.*, No. 33, S. 577, 1897; St. Bondzýnski u. K. Panek, *Ber. d. Deut. chem. Gesellsch.*, Jg XXXV, S. 2959, 1902; St. Bondzýnski, St. Dombrowski u. K. Panek, *Zeitsch. f. physiol. Chem.*, Bd. XLV, S. 83, 1905; F. Pregl, *Pflüger's Archiv.*, Bd. LXXV, S. 87, 1899.

11. Max Cloetta, *Archiv. f. exper. Path. u. Pharmak.*, Bd. XL, S. 29, 1897.

12. O. Thiele, *Zeitsch. f. physiol. Chem.*, Bd. XXXVII, S. 251, 1903.

1. Methods in which the residue left upon evaporation of a certain definite volume of urine is effected by fusing with a mixture of sodium carbonate and saltpetre. These are merely applications to the urine of Liebig's original method of sulphur estimation. Full directions as to the procedure are given in Savelieff's[1] and Moreigne's[2] papers.

2. Methods employing concentrated fuming nitric acid as the oxidising agent. Schulz[3] and Mohr[4] have described methods utilising this reagent. Schulz originally carried out the process in a special closed apparatus, believing that volatile sulphur compounds might be formed, but in his last communication on the subject he states that this is not the case, and now carries out the oxidation in an open Kjeldahl flask. Konschegg,[5] who also uses fuming nitric acid, adds a small quantity of potassium nitrate, probably in order to effect more complete oxidation.

3. Methods in which the oxidation is brought about by sodium peroxide. The introduction of this reagent, for the purpose of sulphur estimations, we owe to Hempel.[6] It was subsequently used by Hoehnel,[7] Glaser[8] and by Asbóth,[9] for the estimation of sulphur in organic materials. In a later paper Asbóth,[10] who subsequently employed sodium peroxide for the estimation of sulphur in organic compounds, suggested that the method might also be useful for the determination of the total sulphur in urine.[11] Modrakowski[12] simplified the method by omitting the addition of sodium carbonate to the peroxide, which formed a part of the original process. More recently Folin[13] has modified the Asbóth-

1. Savelieff, *Virchow's Archiv.*, Bd. CXXXVI, S. 197, 1894.

2. Moreigne, *Bull. de la Soc. Chim.* [3], XI, 975, 1894.

3. H. Schulz, *Pflüger's Archiv.*, Bd. LVII, S. 57, 1894; also *ibid.*, Bd. CXXI, S. 114, 1907-8.

4. P. Mohr, *Zeitsch. f. physiol. Chem.*, Bd. XX, S. 556, 1895.

5. A. Konschegg, *Pflüger's Archiv.*, Bd. CXXIII, S. 274, 1908.

6. W Hempel, *Zeitsch. f. anorg. Chem.*, Bd. III, S. 193, 1893.

7. M. Hoehnel, *Archiv. d. Pharm.*, Bd. CCXXXII, S. 225, 1894.

8. C. Glaser, *Chemiker Zeitung*, Jg. XVIII, S. 1448, 1894.

9. A. von Asbóth, *Chemiker Zeitung*, Jg. XIX, S. 599, 1895.

10. A. von Asbóth, *ibid.*, Jg. XIX, S. 2040, 1895; A. Edinger recommended sodium peroxide for the estimation of organically combined sulphur before Asbóth's paper appeared. He used an aqueous solution of the peroxide. *Zeitsch. f. analyt. Chem.*, Bd. XXXIV, S. 366, 1895; *Ber. d. Deut. chem. Gesellsch.*, Jg. XXVIII, S. 427, 1895.

11. S. Lang used the Asbóth method to estimate total sulphur in urine, but gives no control analyses. *Zeitsch. f. physiol. Chem.*, Bd. XXIX, S. 305, 1900.

12. G. Modrakowski, *Zeitsch. f. physiol. Chem.*, Bd. XXXVIII, S. 561, 1903. F. Clark, *Journ. Chem. Soc.*, 1893, I, p. 1093, had previously suggested that sodium carbonate was unnecessary. Almost immediately after Modrakowski's paper, Neumann and Meinertz (*Zeitsch. f. physiol. Chem.*, Bd. XLIII, S. 38, 1904) advised the admixture of potassium carbonate.

13. O. Folin, *Journ. of Biolog. Chem.*, Vol. I, p. 155, 1906. See also T. B. Osborne, *Journ. of Am. Chem. Soc.*, Vol. XXIV, p. 142, 1902; J. A. Le Clerq and Dubois, *ibid.*, Vol. XXVI, p. 1108, 1904; W. L. Dubois, *ibid.*, Vol. XXVII, p. 729, 1905.

Modrakowski method in several particulars (especially with regard to the addition of a little water before the final fusion in order to obtain complete fusion with the aid of comparatively little heat, and to protect the crucible against corrosion). In all these methods the fusion is accomplished by heat applied to the exterior of the crucible, preferably by means of a methylated spirit burner.

Pringsheim, however, utilising an observation of Parr's,[1] showed that the oxidation may be most easily brought about by the introduction of a red-hot iron nail into the mixture. He first employed this method for the estimation of the halogens, phosphorus and arsenic,[2] and later, following Konek[3] and other observers,[4] to the estimation of sulphur[5] in organic combination. Recently, Abderhalden and Funk[6] have applied Pringsheim's method to the estimation of total sulphur in the urine.

It is obvious that in any trustworthy method, the results obtained with the same urine should agree *inter se*; and in the comparison of different methods, the method which gives the highest results will in the absence of other indications be the most correct. On both these points there are differences of opinion with regard to the different methods: thus Österberg and Wolf[7] state that while the method of Asböth-Modrakowski, carried out according to Folin's directions, invariably gives higher figures than the Schulz method, the results obtained by the latter method do not agree well *inter se*; Konschegg states that his modification of Schulz's procedure gives considerably higher figures than the latter; Abderhalden and Funk claim that the Pringsheim modification in their hands also gives higher figures than Schulz's method; and Gill and Grindley[8] say that Konschegg's figures are invariably higher than those obtained by both Osborne's and Folin's modifications.

Taking all these statements into consideration, and bearing in mind that no corroborative observations have yet appeared on the Pringsheim method, it seemed advisable to investigate the degree of accuracy of the various methods in a series of urines.

1. S. W. Parr, *Journ. Am. Chem. Soc.*, Vol. XXII, p. 646, 1900.
2. H. H. Pringsheim, *Ber. d. Deut. chem. Gesellsch.*, Jg. XXXVI, S. 4244, 1903; *Amer. Chem. Journ.*, Vol. XXXI, p. 386, 1904; *Zeitsch. f. angewand. Chem.*, Bd. XVII, S. 1454, 1904.
3. F. von Konek, *Zeitsch. f. angewand. Chem.*, Bd. XVI, S. 516, 1903.
4. C. Sundstrom, *Journ. of Am. Chem. Soc.*, Vol. XXV, p. 184, 1903; J. D. Pennock and D. A. Morton, *ibid.*, Vol. XXV, p. 1265, 1903.
5. H. H. Pringsheim, *Ber. d. Deut. Chem. Gesellsch.*, Jg. XLI, S. 4267, 1908.
6. E. Alderhalden and C. Funk, *Zeitsch. f. physiol. Chem.*, Bd. LVIII, S. 331, 1908.
7. E. Osterberg and C. G. L. Wolf, *Biochem. Zeitsch.*, Bd. IX, S. 307, 1908.
8. F. W. Gill and H. S. Grindley, *Proceed. of Soc. of Biolog. Chem.*, Vol. VI, p. 11, May, 1909.

In comparing the different methods, I have taken as my standard of agreement the one adopted by Abderhalden and Funk, namely, variations of 0·1 milligr. on either side of the mean value, when the sulphur is estimated in 10 c.c. of urine.

As there seemed to be no necessity of confirming the correctness of the old Liebig method,[1] I limited myself to the following, viz.:—those of Schulz, Konschegg, Asbóth-Modrakowski, and Pringsheim (Abderhalden-Funk modification).

Schulz's Method. The following are the analytical results I have obtained with four different urines:

URINE A

5 c.c. urine gave	0·0358 gr. $BaSO_4$	=	0·0098 gr. sulphur in 10 c.c. urine
,, ,, ,,	0·0362 gr. ,,	=	0·0100 gr. ,, ,, ,,

URINE B

5 c.c. urine gave	0·0348 gr. $BaSO_4$	=	0·0096 gr. sulphur in 10 c.c. urine
,, ,,	0·0353 gr. ,,	=	0·0097 gr. ,, ,, ,,

URINE C

5 c.c. urine gave	0·0510 gr. $BaSO_4$	=	0·0140 gr. sulphur in 10 c.c. urine
,, ,, ,,	0·0501 gr. ,,	=	0·0138 gr. ,, ,, ,,

URINE D

5 c.c. urine gave	0·0154 gr. $BaSO_4$	=	0·0042 gr. sulphur in 10 c.c. urine
,, ,, ,,	0·0150 gr. ,,	=	0·0041 gr. ,, ,, ,,

It will be seen that (contrary to Osterberg and Wolf's statement) the figures obtained agree very well *inter se*, the maximum variation being only 0·2 milligr. Mohr, who used a method almost identical with that of Schulz, also obtained concordant results. My results by the Schulz method are, however, lower than those obtained by other methods, as will be immediately seen.[2]

Konschegg's Method. The following are my analytical figures:—

URINE A

5 c.c. urine gave	0·0378 gr. $BaSO_4$	=	0·0104 gr. sulphur in 10 c.c. urine
,, ,, ,,	0·0375 gr. ,,	=	0·0103 gr. ,, ,, ,,

URINE B

5 c.c. urine gave	0·0369 gr. $BaSO_4$	=	0·0101 gr. sulphur in 10 c.c. urine
,, ,, ,,	0·0384 gr. ,,	=	0·0106 gr. ,, ,, ,,

URINE C

5 c.c. urine gave	0·0528 gr. $BaSO_4$	=	0·0145 gr. sulphur in 10 c.c. urine
,, ,, ,,	0·0524 gr. ,,	=	0·0144 gr. ,, ,, ,,

URINE D

5 c.c. urine gave	0·0165 gr. $BaSO_4$	=	0·0045 gr. sulphur in 10 c.c. urine
,, ,, ,,	0·0163 gr. ,,	=	0·0045 gr. ,, ,, ,,

1. See however O. Folin, *Journ. of Biolog. Chem.*, Vol. I, p. 156, 1906.

2. This confirms the statement of Konschegg, Osterberg and Wolf, Abderhalden and Funk (*loc. cit.*) and of Sherman who used the nitric acid method for the estimation of sulphur in organic compounds. (*Journ. of the Amer. Chem. Soc.* Vol. XXIV, p. 1100, 1902).

Pringsheim's Method (Abderhalden and Funk's modification). The following are my analytical figures:—

URINE A

10 c.c. urine gave 0·0740 gr. $BaSO_4$ = 0·0102 gr. sulphur in 10 c.c urine
„ „ „ 0·0744 gr. „ = 0·0102 gr. „ „ „

URINE B

10 c.c. urine gave 0·0798 gr $BaSO_4$ = 0·0110 gr. sulphur in 10 c.c. urine
„ „ „ 0·0800 gr. „ = 0·0110 gr. „ „ „

URINE C

10 c.c. urine gave 0·1032 gr. $BaSO_4$ = 0·0142 gr. sulphur in 10 c.c. urine
„ „ „ 0·1043 gr. „ = 0·0143 gr. „ „ „

URINE D

10 c.c. urine gave 0·0322 gr. $BaSO_4$ = 0·0044 gr. sulphur in 10 c.c. urine
„ „ „ 0·0318 gr. „ = 0·0044 gr. „ „ „

URINE E

10 c.c. urine gave 0·0756 gr. $BaSO_4$ = 0·0104 gr. sulphur in 10 c.c. urine
5 c.c. „ „ 0·0383 gr. „ = 0·0106 gr. „ „ „

It will be seen, in confirmation of what Abderhalden and Funk state, that the figures are very concordant.

If the figures obtained by the Konschegg method are compared with the above obtained by the Pringsheim method, it will be observed that the Konschegg figures are a little higher (1 to 2·1 per cent.) than those obtained by this method, and 5·2 per cent.[1] on the average higher than those obtained by Schulz's method.

Asbóth-Modrakowski Method. Here different results were obtained, according to differences in the details of the *modus operandi*. In most of my estimations I carried out the process in the following manner:—I added the urine gradually to the sodium peroxide to avoid loss by spitting,[2] evaporated down on the water bath to complete dryness, and subjected the residue to complete fusion over a spirit burner, maintaining it in the fused condition for ten to fifteen minutes; cooled; added 1-2 c.c. of distilled water, and more peroxide and again subjected the mixture to complete fusion, prolonging the fusion for twenty to thirty minutes. I used between three and four grams of sodium peroxide for the preliminary fusion and eight grams for the final; the quantity of urine used was 25 c.c. Using this method I obtained, in all the estimations I performed, agreement between the individual determinations made on the same urine, and also higher results than by any of the other methods.[3] On the average the figures were 8 per cent. higher than those

1. This difference is much less than that given by Konschegg himself.
2. Recommended by Modrakowski, *loc. cit.*
3. M. A. Desmoulière, *Journ. de Pharm. et de Chim.* [6th series], Tome XXIV, p. 294, 1906, states that Moreigne's and Modrakowski's methods give accurate results in estimation of total sulphur in urine; the single comparison of figures which he gives for the two methods shows a slight excess in favour of Modrakowski. This, taken in conjunction with Abderhalden and Funk's demonstration, that the Pringsheim method gives the same figures (really slightly lower) as the $Na_2CO_3-KNO_3$ method, accords with my results.

given by the Konschegg method. I was unable to detect any evolution of sulphuretted hydrogen, either during the evaporation or the subsequent acidification of the fused mass, such as Gill and Grindley describe.

The following are my analytical figures:—

URINE B

25 c.c. urine gave 0·2036 gr. $BaSO_4$ = 0·0112 gr. sulphur in 10 c.c. urine
„ „ „ 0·2053 gr. „ = 0·0113 gr. „ „ „

URINE C

25 c.c. urine gave 0·2724 gr. $BaSO_4$ = 0·0150 gr. sulphur in 10 c.c. urine
„ „ „ 0·2798 gr. „ = 0·0154 gr. „ „ „

URINE D

25 c.c. urine gave 0·0966 gr. $BaSO_4$ = 0·0053 gr. sulphur in 10 c.c. urine
„ „ „ 0·0970 gr. „ = 0·0053 gr. „ „ „

This method, as I have already described it, is in the main identical with that described by Folin, except that he does not mention the preliminary fusion, and maintains that ten minutes' final fusion is sufficient. The number of analyses I have carried out according to his directions is not great; they agree well *inter se*, but they are never higher than those obtained by Pringsheim's method, and in the one case where I can make the comparison they are lower than those obtained by Konschegg's method.[1] The following are my analytical figures:—

URINE A

25 c.c. urine gave 0·1860 gr. $BaSO_4$ = 0·0102 gr. sulphur in 10 c.c. urine
„ „ „ 0·1858 gr. „ = 0·0102 gr. „ „ „

URINE E

25 c.c. urine gave 0·1858 gr. $BaSO_4$ = 0·0102 gr. sulphur in 10 c.c. urine
„ „ „ 0·1880 gr. „ = 0·0103 gr. „ „ „

Putting all the results together, the following table shows the mean figures expressed in grams of sulphur per 10 c.c. urine.

Method	Urine A	Urine B	Urine C	Urine D	Urine E
Schulz	0·0099	0·0097	0·0139	0·0042	—
Konschegg	0·0104	0·0104	0·0145	0·0045	—
Pringsheim	0·0102	0·0110	0·0143	0·0044	0·0105
Asbóth-Modrakowski	—	0·0113	0·0152	0·0053	—
Asbóth-Modrakowski (Folin) ...	0·0102	—	—	—	0·0103

From the above table it will be seen that Schulz's method gives the lowest, whilst the Asbóth-Modrakowski method gives the highest figures; the Pringsheim, Konschegg and the Folin's modification of the Asbóth-Modrakowski method all give intermediate figures.

Conclusion. The sodium peroxide method carried out according to Asbóth-Modrakowski (as described above) gives the highest figures in the estimation of the total sulphur in the urine, and must therefore be considered to be the most trustworthy of the methods at present in use.

1. Gill and Grindley, *loc. cit.*, state that the difference is even greater than I have obtained.

THE USE OF BARIUM PEROXIDE IN THE ESTIMATION OF TOTAL SULPHUR IN URINE

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Although the Asbóth-Modrakowski method gives the best results for the estimation of total sulphur in urine (see preceding paper), it has the disadvantage of being somewhat lengthy. In metabolic experiments, where it is necessary to make a large number of estimations, it is essential that a process should be adopted which can be carried out rapidly. From this point of view the Pringsheim method seemed to be the best, provided it could be modified so as to give figures equal to those obtained by the Asbóth-Modrakowski method. With this end in view it occurred to me that the use of barium peroxide, in the fusion of which a higher temperature is obtained than with sodium peroxide, might lead to a more complete oxidation. The introduction of a barium salt would have the additional advantage of shortening the method, as the barium sulphate is formed during the actual process of the oxidation.[1] I therefore tried fusing with barium peroxide instead of sodium peroxide, but found that fusion did not take place easily.

This difficulty was overcome by the addition of sodium peroxide to the barium peroxide in the proportion of seven to one. The process was then carried out exactly as in the Pringsheim method, the details of this new method being as follows:—

1. Applying this idea to the Konschegg method, I found that the addition of barium nitrate does not give any better results than when it is absent. The following are my mean figures, given in grammes of sulphur per 10 c.c. urine:—

Method	Urine A	Urine B	Urine C	Urine D
Konschegg	0·0104	0·0104	0·0145	0·0045
Barium-nitrate-nitric acid ...	0·0102	0·0109	0·0144	0·0042

The addition of barium peroxide in the Asboth-Modrakowski method did not yield concordant results, and as the method is too lengthy I did not pursue it further.

In the literature the only references I could find with regard to the utilisation of a barium salt in the estimation of sulphur were:—

(*a*) H. Weidenbusch, who, at Liebig's suggestion, estimated sulphur in albuminous materials by fusing a paste formed of the substance, barium nitrate and nitric acid. He gives no control analyses. *Liebig's Ann.*, Bd. LXI, S. 370, 1847.

(*b*) Alb. Edinger used barium peroxide to estimate sulphur in inorganic and organic substances, finally fusing with sodium peroxide. *Zeitsch. f. analyt. Chem.*, Bd. XXXIV, S. 366, 1895.

Ten c.c. of urine were measured into a nickelled steel crucible, as recommended by Pringsheim,[1] and made alkaline with sodium carbonate. After the addition of 0·4 grm. lactose, the mixture was evaporated down, on the water-bath, to a syrupy residue. Without further drying, the residue was carefully mixed with 8 grms. of the oxidising agent, consisting of 7 grms. of sodium peroxide and 1 grm. of barium peroxide.[2]

The crucible is next immersed up to three-quarters of its height in distilled water contained in a larger porcelain crucible or basin. A red-hot iron nail is introduced through the hole in the lid, and in a few seconds the reaction is completed. When the crucible has cooled down sufficiently it is overturned into the water, the basin being covered by a clock glass. The contents of the basin are then transferred quantitatively into a 500 c.c. Erlenmeyer flask and raised to boiling point. Concentrated hydrochloric acid is added gradually to the boiling fluid until the ferric oxide (derived from the iron nail) has gone into solution. A small excess of hydrochloric acid and a few c.c. of alcohol are then added, and the boiling continued for a short time. This serves to drive off the chlorine, which is always formed by the action of the excess of sodium peroxide on acidifying the solution with hydrochloric acid. It was noticed that under the above conditions the oxidation (as judged by the absence of carbonaceous particles) is complete, whilst without the use of barium peroxide filtration is very frequently essential in order to remove particles of carbon. The barium sulphate which is now present in the form of a granular precipitate is then collected on a weighed Gooch crucible, dried, ignited, and weighed as usual. It was found as a further advantage of the barium peroxide addition that the barium sulphate precipitate settles and filters very easily, probably owing to the physical conditions under which it is formed.

Using this method my analytical figures were the following:—

Urine B

10 c.c. urine gave	0·0857 gr. $BaSO_4$	=	0·0118 gr. sulphur in 10 c.c. urine
,, ,, ,,	0·0837 ,,	=	0·0115 ,, ,, ,,

Urine C

10 c.c. urine gave	0·1112 gr. $BaSO_4$	=	0·0153 gr. sulphur in 10 c.c. urine
,, ,, ,,	0·1122 ,,	=	0·0154 ,, ,, ,,

1. The crucible, together with the perforated lid, is obtainable from Messrs. Köhler, Leipzig.

2. A certain amount of care must be exercised in the addition of the oxidising agent. I found it advisable to add about 0·1 to 0·2 gramme at a time at the beginning. After the addition of about half the required amount, the remainder may be added in larger quantities. The barium peroxide used was tested for sulphur with negative results.

Urine D

10 c.c. urine gave	0·0472 gr. $BaSO_4$	=	0·0065 gr. sulphur in 10 c.c. urine				
" " "	0·0488 "	=	0·0067 " " "				

From this it will be seen that the results agree well *inter se.* If we compare the mean figures obtained by this method with those obtained without the use of barium peroxide (Pringsheim method) it will be seen to give considerably higher results. Compared with the Asbóth-Modrakowski method the results are a little higher, showing that under these conditions the oxidation is even more complete than in the Asbóth-Modrakowski method.

	Urine B	Urine C	Urine D
Pringsheim method	0·0110	0·0143	0·0044
Asbóth-Modrakowski method ...	0·0113	0·0152	0·0053
New method	0·0117	0·0154	0·0066

It follows, therefore, that the estimation of total sulphur in urine by means of a mixture of barium and sodium peroxides gives the highest results and possesses the further advantage of being carried out rapidly.

I have much pleasure in acknowledging with thanks the constant help and encouragement I have received from Dr. Rosenheim during the progress of these experiments.

A CONTRIBUTION TO THE BIO-CHEMISTRY OF HAEMOLYSIS:—

(a) CHANGES IN SOLUBILITY OF THE LIPOIDS IN PRESENCE OF ONE ANOTHER, AND OF CERTAIN UNSATURATED ORGANIC SUBSTANCES.

(b) THE BALANCING ACTION OF CERTAIN PAIRS OF HAEMOLYSERS IN PREVENTING HAEMOLYSIS.

(c) THE PROTECTIVE ACTION OF SERUM PROTEINS AGAINST HAEMOLYSERS.

(d) THE EFFECTS OF OXYDISING AND REDUCING AGENTS UPON HAEMOLYSIS.

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The subject of haemolysis, and the relationship of lipoid substances to this process of laking of the blood corpuscles, is one which is at the present time exciting very general attention from physical chemists, biological chemists, and clinicians alike because of its important relationships to the chemistry of colloidal solutions on the one hand, and of its valuable applications to the diagnosis of disease on the other.

In earlier papers more directly concerned with the subject of the digestion and absorption of fats, it was shown in 1897 by Moore and Rockwood[1] and in 1901 by Moore and Parker[2] that the salts of the bile, and the products of fatty cleavage of a lipoidal nature, possessed when in common solution some kind of an affinity, apparently of a physico-chemical nature, which had the effect of increasing the solubilities of the fatty acids and soaps.

This remarkable change in solubilities was shown by Moore and Parker to extend to other lipoids, such as lecithin. It was further demonstrated that the unsaturated oleic acid and its sodium soap had a

1. *Proc. Roy. Soc.*, Vol. LX, p. 438, 1897; *Journ. of Physiology*, Vol. XXI, p. 58, 1897.
2. *Proc. Roy. Soc.*, Vol. LXVIII, p. 64, 1901.

most peculiar effect in increasing many times the solubilities of the fully saturated palmitic and stearic acids and their sodium soaps. These when in a state of purity were found to have practically a zero solubility in either water or bile salt solution.

Moore and Parker showed in the case of lecithin, which they prepared from egg yolk, that the lecithin, when it was added to a solution of bile salts or to bile at body temperature, did not form an emulsion or fine suspension as in the case of treatment with water, but gave instead a water clear solution. This solution was then more effective than the original in dissolving other lipoids.

They also found, probably on account of this mutual effect upon solubilities of the different lipoids, that bile as a whole was a much more effective solvent than a considerably stronger solution of the separated and re-dissolved bile salts.

These earlier results on mutual solubility appear to us to possess a bearing, which will be pointed out later, upon the process of haemolysis of the lecithin-containing corpuscles by other lipoids, such as sodium oleate, the bile salts, and saponin-like bodies, and for this reason we quote here certain of the figures given by Moore and Parker which definitely show the mutual effects.

The solubility of the fatty acids and soaps was found to be as follows:—

'*Oleic Acid:* solubility in distilled water less than 0·1 per cent.; solubility in 5 per cent. bile salt solution, about 0·5 per cent.; solubility in 5 per cent. bile salts *plus* one per cent. lecithin, 4·0 per cent.'

'*Palmitic Acid:* in distilled water less than 0·1 per cent.; in 5 per cent. bile salts, about 0·1 per cent.; in 5 per cent. bile salts *plus* 1 per cent. lecithin, 0·6 per cent.'

'*Stearic Acid:* in distilled water less than 0·1 per cent.; in 5 per cent. bile salts less than 0·1 per cent.; in 5 per cent. bile salts *plus* 1 per cent. lecithin, 0·2 per cent.'

'*Sodium Oleate:* in distilled water, 5·0 per cent.; in 5 per cent. bile salts, 7·6 per cent.; in 5 per cent. bile salts *plus* 1 per cent. lecithin, 11·6 per cent.

'*Sodium Palmitate:* in distilled water, 0·2 per cent.; in 5 per cent. bile salts, 1·0 per cent.; in 5 per cent. bile salts *plus* 1 per cent. lecithin, 2·4 per cent.'

'*Sodium Stearate:* in distilled water, 0·1 per cent.; in 5 per cent. bile salts, 0·2 per cent.; in 5 per cent. bile salts *plus* 1 per cent. lecithin, 0·7 per cent.'

'*Lecithin.* "Pure" lecithin is practically insoluble in water, the addition of as little as 0·1 per cent. causes an opalescence and further additions give rise, as is well known, to a kind of emulsion. But when lecithin is added to a 5 per cent. solution of bile salts, or to bile, the appearances observed are quite different.'

'The lecithin dissolves to a clear brown-coloured solution and the amount taken up is surprising: thus a 5 per cent. solution of bile salts takes up no less than 7 per cent. of lecithin

at a temperature of 37° C. On cooling, part of the lecithin is thrown out of solution as a finely suspended precipitate or emulsion which glistens with a silky lustre when the test-tube containing it is shaken so as to set the fluid in motion. At ordinary room temperatures of 15° to 20° C. a considerable amount of lecithin, 4 to 5 per cent., is, however, still retained in solution.'

'The power of lecithin in increasing the solubilities of the fatty acids and soaps, explains in great part why lower solubilities are obtained in experimenting with pure bile salt solutions, than with bile. The lecithin naturally occurring in bile thus increases the solvent power of that fluid in the intestine for fatty acids and soaps.'

We have quoted at length these earlier experiments upon the mutual effects of different lipoids in common solution upon one another, because they appear to us to have some bearing upon haemolytic phenomena. For example, sodium oleate or sodium linoleate have a strong laking effect upon the red blood corpuscles. Now the red blood corpuscles contain lecithin, but the above experiments show that the presence of lecithin in solution increases the solubility of oleates. In haemolysis of this type it is hence obvious that the converse result is being obtained and that the oleates or linoleates are laking the corpuscles, *because* lecithin is more soluble in presence of the oleates or linoleates.

We shall also see that the bile salts and the members of the saponin-digitalin group of glucosides are all unsaturated compounds like the oleates and linoleates, and that they increase by their presence lipoid solubilities, and hence are powerful laking agents.

These results upon solubility were confirmed and extended in several papers by Pflüger[1] and others, and Pflüger laid particular stress upon the effect of the presence of sodium carbonate and of oleic acid and oleates in raising the solubilities of the other constituents.

The above experiments upon solubility of lipoid materials and their derivatives may now be considered in relationship to haemolysis.

A very considerable portion of the stroma of the red corpuscle is lipoidal in character, that is to say, is soluble in ether or similar solvents. The amount is placed at one-third of the dry weight by Pascucci,[2] and of this a large amount consists of mixed lecithides, containing unsaturated fatty acids in the molecule.

Accordingly, any constituent in a serum or suspending saline which possesses the property of increasing the solubility of these lecithides must tend to lake the corpuscles by dissolving up the stroma. Such an action, as shown by Moore and Parker, is possessed by the bile salts, and they accordingly act as powerful haemolysers.

1. *Arch. f. d. ges. Physiol.* Bd. LXXXII, 1900, S. 303, 381; LXXXV, 1901, S. 1; LXXXVIII, 1902, S. 299, 431; XC, 1902, S. 1.

2. *Hofmeister Beiträge*, 1905, Vol. VI, p. 543; Iscovesco ('Les Lipoides.' p. 13, 1908) places the amount of lipoids in the dried corpuscle at a lower value than one-third.

This haemolytic power of the bile has long been known qualitatively; it has just now been followed out quantitatively in this laboratory by MacLean and Hutchinson with the most interesting results, recorded in the paper immediately succeeding this one.[1]

In the same fashion, we have seen that oleic acid and oleates were found experimentally to raise the solubilities of the practically insoluble palmitates and stearates in the presence of bile salts. Also, even in the absence of all bile derivatives, the solubilities in water obtained by Moore and Parker for the separate sodium salts of the acids, oleic, palmitic, and stearic on the one hand, and for the mixed sodium soaps of naturally occurring fats of pig, ox and sheep on the other, clearly show that the presence of sodium oleate increases the solubility of the other soaps.

These experimental results must be the basis of the results obtained by many observers that sodium oleate is a powerful haemolyser, while, as demonstrated by Noguchi,[2] the sodium palmitate and sodium stearate are inert.

A chemical point of great importance is that both the oleic acid and the bile acids are unsaturated bodies containing in each case doubly-linked carbon atoms in an open chain, and this suggests the general law, first enunciated by St. Faust and Tallqvist,[3] that the haemolytic property is associated with this absence of saturation.

At the outset of our work, we were unfortunately unaware of the existence of St. Faust and Tallqvist's paper, and we must express our regret that for this reason we were unable in a preliminary communication[4] to do justice to their most interesting work upon the subject.

These authors, in following out in a highly interesting fashion the causes of a pernicious anaemia due to the intestinal parasite, *Botriocephalus latus*, were able to separate from the dried bodies of the parasites a material consisting to a large extent of an unstable compound of cholesterin and oleic acid. This substance was shown to be a cholesterin ester of oleic acid of the type first separated from blood serum by Hürthle.[5] This cholesterin-oleic ester had a most powerful haemolytic effect even in small quantities, and on further testing the matter, St. Faust and Tallqvist discovered that the haemolytic action was due to the oleic acid, and that sodium oleate gave a like result, while saturated soaps or their esters gave no effect upon the blood corpuscles.

1. See page 369.
2. Noguchi, *Journ. of exper. Medicine*, Vol. VIII, p. 92, 1906.
3. *Arch. f. exper. Path. u. Pharm.*, Vol. LVII, p. 370, 1907.
4. *Journ. of Physiol.*, Proc. Physiological Society, March, 1909.
5. *Zeitsch. f. Physiol. Chem.*, Bd. XXI, 1895-6, S. 331.

This result led the authors to the generalisation that such haemolytic action was associated with the want of saturation of the oleic acid. This was tested by employing other unsaturated acids such as acrylic, tiglic, cinnamic and erucic acids, and it was found in each case with the free acids there was marked haemolysis, although in the case of the sodium salts of tiglic and cinnamic acids there was no haemolytic activity. St. Faust and Tallqvist further demonstrated in support of their view, that when acrylic acid is hydroxylated into hydracrylic acid the latter is almost without action upon blood corpuscles.

Now one of these acids has a double bond which has been split up in the other, as shown by the formulae given below, and this is probably the cause of the difference in activity.

Acrylic acid	Hydracrylic acid
CH_2	CH_2OH
‖	|
CH	CH_2
|	|
$COOH$	$COOH$
Strongly haemolytic	Not haemolytic

We have ourselves been able in the present series of experiments to demonstrate that an unsaturated glucoside with strongly haemolytic properties, isolated by Moore from the seeds of *Bassia longifolia* (Mowrah seeds), called 'Mowrin,' loses its haemolytic properties when it becomes saturated by bromination.

It is difficult to explain why the sodium salts of tiglic and cinnamic acids do not haemolyse, for they are unsaturated compounds. It may be that a certain conformation of molecule in addition to the double linkage is necessary in order to confer upon the haemolytic molecule certain physico-chemical properties which we shall subsequently see all these haemolysers possess, and that the want of saturation really confers laking power because of the physical properties of solution, etc., which attach themselves to it, and not because the double bond is broken to allow a firm combination between the two compounds.

St. Faust and Tallqvist do not appear to have tested other acids and salts than those mentioned above, nor to have proceeded to the further generalisation that a similar lack of saturation characterises other laking agents such as the bile salts and saponins.

Believing that the view is one of somewhat far-reaching importance, we have in the present experiments tested it with a number of haemolytic substances, and have always found that substances of this nature which were haemolytic were also unsaturated or possessed of a good deal of residual chemical affinity.

Connected with the above two points of haemolytic power and want of chemical saturation, these bodies—often of widely different origin and chemical constitution—possess always a well-marked group of common properties, physical and physiological, which are so striking when placed in juxtaposition as to indicate that they and the laking process are all closely connected together and have a common cause.

Further, these common properties, which will presently be stated, are such that in spite of the fact of chemical unsaturation running through the whole group, it is difficult, or indeed impossible, to draw any definite conclusion as to whether these bodies act by forming a feeble labile chemical union, or by physically altering the properties of the solvent so that it now dissolves the lipoids.

The energy phenomenon at play is evidently one due to interaction between dissimilar chemical molecules, but whether it consists wholly, or as an initial stage, in a lowering of the surface tension at the interface between lipoid and solvent, or whether there is first a labile chemical union between lipoid and unsaturated acid causing an accumulation on the interface, so leading to a negative surface tension and hence to solution of the lipoid and to haemolysis, it is impossible to say in the present state of our knowledge.

It may be remarked, however, that the combating physical and chemical hypotheses are not so very widely apart as the two camps of adherents suppose, for in either case, the interaction is between dissimilar molecules or aggregates at an interface, and this does not differ widely from chemical action. We do not know what are the initial 'physical' stages of 'chemical' combination.

It might be asked, what is the nature of the energy change which causes accumulation of a dissolved substance on an interface and lowering of surface tension, if it be not chemical attraction and the preliminary stage of a chemical reaction? Leaving these more abstract considerations of chemical combination *versus* physical adsorption, we may now return to the characteristic chemical and physiological properties of the group of haemolytic agents which we are discussing.

Properties of Haemolysers

Physical Properties.—All these substances are colloidal in aqueous solution, although some of them diffuse very slowly through parchment paper; they do not crystallise out of aqueous solution, and they give rise to thick syrups as they are evaporated down to more and more concentrated

solutions. Even in dilute solution they all froth easily, showing probably that the surface-tension is lowered.[1]

Chemical Properties. All show a great tendency to form conjugated compounds, which are very easily hydrolysed by dilute acids. For example, the bile salts with amino-acids, such as glycocoll or taurin; oleic acid with cholesterin to form cholesterin esters, and with glycerol to form fats; saponin, digitalin, mowrin, and other haemolysers of that type are glucosides; the lecithides are not only conjugated compounds themselves, but unite in feeble union with a vast number of substances of biological origin, such as snake venoms and tox-albumens.

This property of conjugating chemically is, as we shall see, of the utmost importance in connection with haemolysis, where it also occurs, and may cause active haemolysis or an anti-phase, according to how it is directed.

Physiological Properties. The physiological properties of the whole group are closely related, and are, no doubt, dependent upon the above physical and chemical properties. Thus the soaps, the bile salts, and the whole saponin-digitalin group, are characterised by a very intensely bitter taste. Introduced directly into the circulation, they are all poisonous, and all affect the heart, causing slowing and stopping. This is in all probability due to a common cause, viz., combination with the heart lecithides.

That same physico-chemical property which attacks the red blood corpuscles by means of the attraction for its lecithin and causes haemolysis, causes attack, always of a common type with minor variations, upon the heart, due here also to chemical attraction between soap (sodium oleate), bile salt, saponin, mowrin, digitalin, or what not, of this large group of unsaturated bodies on the one hand, and the heart lecithide on the other. So variations in reactivity are caused within the heart cell, and accompanying modifications in heart beat. Here it is to be remarked that it is an integral change inside the cell of which the lecithide is a vital part that occurs, and is not a mere question of altered permeability of a lipoidal membrane.

These peculiar properties are shown in varying degree by different members of the group, but taken together they form a good set of characteristics for a very widely distributed group of substances all possessing haemolytic properties.

1. This is known to be so with the soaps, experiments with other haemolysers are in progress.

The Balancing Action of Haemolysers

One of the most interesting experiments in haemolysis is that of Sachs and Altmann,[1] demonstrating that two bodies, each of which is strongly haemolytic in itself, can be so admixed in common solution that no haemolysis whatever results, the two haemolysers balancing each other.

Thus, it was found that when sodium oleate was added in just the proper quantity to a strongly active haemolytic serum no haemolysis resulted, and that as the amount of oleate was gradually increased above this balancing amount, the mixture gradually became haemolytic again.

This result has been stated to be due to the neutralising of complement, the sodium oleate acting as an anti-complement. We think, however, that there is clear evidence against this view. In the first place, as we shall see later, an ordinary serum which is not haemolytic to the corpuscles being used, it may, in fact, be their own serum, is strongly protective against the haemolytic action of sodium oleate.

We have followed this question up in detail, as shown by the protocols of our experiments, and have successively removed or destroyed (*a*) immune body, (*b*) complement, and (*c*) the lipoids from the active serum. In all cases we have found that no one alone of these substances is responsible for the neutralizing of the haemolytic activity of the sodium oleate.

The further fact that not only is the laking power of the sodium oleate destroyed, but also the natural activity of the pig's serum, or the invoked activity of a specially sensitized serum, appears to us to clearly demonstrate that the soaps of the unsaturated fatty acids, oleic and linoleic, possess a selective affinity for the immune body, or actively laking, substance, of these haemolytic sera. That is to say, in the active serum the immune body and the sodium oleate or linoleate combine and mutually destroy each the other's laking power, so that the mixture in due proportion is quite inert upon the blood corpuscles.

But in case the immune body has been inactivated by heating, then the sodium oleate or linoleate is still captured and held by the serum proteins, so that no laking occurs until much more of the oleate or linoleate has been added than would have sufficed to cause complete and rapid laking in a saline suspension, where there is no protein to present a counter-attraction and binding agent, so that the first trace of oleate or linoleate at once attacks the lecithides and other lipoids of the corpuscles. When the serum proteins are present, although inactive themselves, they

1. *Berl. klin. Wochenschr.*, pp. 494, 699, 1908.

form binding material for unsaturated lipoids, such as the oleates and linoleates.

The action of lecithin and cholesterin of a similar type can be explained on similar lines rather than on the view that these substances behave as active anti-complements.

On the other hand, as our experiments also prove, two lipoids of nearly allied nature which do not therefore combine with each other, or mutually adsorb each other, such as oleate and linoleate of sodium, show no balancing action whatever, but produce a distinctly additive effect. So that whether shown by the smaller amounts which will produce complete laking in a given time in presence of each other, or better by observing the laking times of two minimal amounts of oleate and linoleate separately, as compared with the time spent for laking with the halves of these amounts acting in consort, the result always comes out that the haemolytic effect consists of the two added factors of oleate action and linoleate action; there being no reduction whatever due to action between the two haemolysers, such as is seen between either of them and the active haemolytic body of a sensitized serum or a serum naturally haemolytic.

We may hence enunciate the law that if two given haemolysers are capable of combining or adsorbing with each other, they will tend to balance each other, and the effect on corpuscles will be less than either acting alone; but if no adsorption is possible between the two, the effect in common solution upon the corpuscles will be the sum of the effects of the two.

Effects of Oxidizing and Reducing Agents upon Haemolysis

These experiments were suggested by analogies between the mode of action of the peroxidases and haemolytic serum, in that heating to 56° C. destroys the tissue peroxides and so stops the action of the peroxidases in a somewhat similar way to that in which heating to 56° C. inactivates a haemolytic serum by destroying complement.

The results of experiment showed that it was not possible to replace the destroyed complement of an inactivated serum by means of hydrogen peroxide or other form of peroxide, so that the haemolytic agent or immune body can hardly be regarded as a peroxidase ferment.

Yet the experiments yielded the very interesting information that addition of an alkaline reducing agent, such as ammonium sulphide, even in very small amount, entirely inactivated an active serum, and contrariwise an oxidizing agent, such as hydrogen peroxide *in alkaline*

solution, very much increased the haemolytic power. The peroxide alone, or hydrogen sulphide alone, in absence of alkali, had very little action; but the addition of a trace of ammonia at once produced the inhibiting action in the case of the sulphide, or favouring action in the case of the peroxide.

In view of the fact that ammonia and other alkalies by themselves possess a laking effect, it may be emphasized that the amounts being used lay below the laking amounts when used alone, as shown by control experiments.

Experimental Methods and Results

The sodium salts of five fatty acids were taken, namely, the sodium salts of stearic, palmitic, erucic, oleic and linoleic acids.

In this list the first two are sodium soaps of saturated fatty acids, belonging to the acetic acid series; sodium erucate and oleate belonging to the acrylic series, are unsaturated sodium soaps, each having one doubly-linked carbon atom in their formula; sodium linoleate belonging to the linolic acid series is still more unsaturated, having three doubly-linked carbon atoms in its constitution.

To test the haemolytic power of these soaps, solutions varying in strength from 0·01 M to 0·001 M were made up by the simple procedure of weighing out the requisite amount of pure free acid and neutralising with the calculated amount of decinormal alkali. Thus, taking $C_{18}H_{34}O_2$ as the formula for oleic acid, this gives a molecular weight of 282, which is equivalent to 0·282 grams in 100 c.c. for a centimolecular solution. This weight of oleic acid was therefore weighed out in a beaker and neutralised with 10 c.c. of 0·1 M NaOH, and the volume made up to 100 c.c. by adding normal isotonic saline solution. The weaker molecular strengths were made up by adding proportionally more normal saline. The other sodium soaps were made up in a similar manner.

For the experiment a series of test-tubes were taken, and in each was placed a certain known quantity of the sodium soap, whose haemolytic properties it was desired to test, and 1 c.c. of a 5 per cent. emulsion of sheep's red blood corpuscles; the volume of each tube was then made up to 5·5 c.c. by adding normal isotonic saline solution. When the contents of the tube were completed, they were placed in a thermostat at a temperature of 37° C. and observations made.

It should be mentioned that in this and the subsequent experiments the emulsion of sheep's red blood corpuscles was made by defibrinating

fresh blood and then washing and centrifuging the corpuscles three times in normal saline, and finally the washed red blood corpuscles were made up into a 5 per cent. emulsion in normal isotonic saline.

The results of several experiments with the soaps above mentioned gave the following results:—

In the tubes containing:

0·2 c.c. $\frac{M}{100}$	sodium stearate.	No haemolysis in 24 hours.
0·2 c.c. $\frac{M}{100}$	sodium palmitate.	No haemolysis in 24 hours.
0·2 c.c. $\frac{M}{100}$	sodium erucate.	Complete haemolysis in 24 hours.
0·8 c.c. $\frac{M}{1000}$	sodium erucate.	No haemolysis in 24 hours.
0·2 c.c. $\frac{M}{100}$	sodium oleate.	Complete haemolysis in 1 hour.
0·4 c.c. $\frac{M}{1000}$	sodium oleate.	Complete haemolysis in 3 hours.
0·2 c.c. $\frac{M}{1000}$	sodium oleate.	Slight amount of laking after 20 hours.
0·4 c.c. $\frac{M}{4000}$	sodium oleate.	Merest trace of laking after 20 hours.
0·2 c.c. $\frac{M}{100}$	sodium linoleate.	Complete haemolysis in 16 minutes.
0·8 c.c. $\frac{M}{1000}$	sodium linoleate.	Complete haemolysis in 50 minutes.
0·4 c.c. $\frac{M}{1000}$	sodium linoleate.	Complete haemolysis in 153 minutes.
0·2 c.c. $\frac{M}{1000}$	sodium linoleate.	Complete haemolysis in 20 hours.
0·4 c.c. $\frac{M}{4000}$	sodium linoleate.	Slight amonnt of laking in 24 hours

It will be seen that in the case of the saturated sodium soaps of stearic and palmitic acid no haemolysis was observed with the above strengths, but with very much weaker strengths of the unsaturated soaps complete haemolysis was obtained, and that sodium linoleate, which has the greater number of doubly-linked carbon atoms, possesses also the strongest laking action, completely haemolysing the sheep's red blood corpuscles within twenty hours, even in a concentration equivalent to 0·000004 M. Sodium oleate, which has one doubly-linked carbon atom, is also a powerful haemolytic agent, but not so active as sodium linoleate, though more powerful than sodium erucate, which is an equally unsaturated soap, but with a different molecular constitution, and with corresponding physical

properties showing less typically the common character of the class of haemolysers.

On the same grounds, some observations were also made with a glucoside mowrin and the sodium salt of mowric acid, one of the products prepared from the glucoside by hydrolysis. These preparations, which are unsaturated bodies, were prepared by Moore from the seeds of *Bassia longifolia*, commonly known as Mowrah seeds.

The following are the haemolytic results obtained with these substances, using 1 c.c. of 5 per cent. emulsion of sheep's red blood corpuscles and a total volume of 5·5 c.c., and following the same technique as in the previous experiments:—

1 c.c.	$\frac{M}{100}$	Mowrin.	Complete haemolysis within 1 minute.
0·75 c.c.	$\frac{M}{10000}$	,,	Complete haemolysis within 4 hours.
0·5 c.c.	$\frac{M}{10000}$	,,	No haemolysis in 24 hours.
1 c.c.	$\frac{M}{100}$	Sodium Mowrate.	Complete haemolysis in 15 minutes.
1 c.c.	$\frac{M}{1000}$	,, ,,	Trace of haemolysis in 20 hours.
0·75 c.c.	$\frac{M}{1000}$	,, ,,	No haemolysis.

It will thus be seen again that these unsaturated bodies, especially the glucoside mowrin, are also powerful haemolytic bodies.

If, however, the sodium salt of mowric acid is brominated, the haemolytic action is markedly weakened, for instance, 1 c.c. of 0·01 M sodium mowrate haemolyses in fifteen minutes, but of an exactly similar quantity if the brominated sodium mowrate be used, the time required in this case for complete haemolysis is five hours.

In view of the fact that there are present in the normal organism many unsaturated haemolytic lipoids, it is of interest to note the protective action that the animal's serum is able to exert on behalf of its own red blood corpuscles.

In order to study this action as regards the three unsaturated haemolytic soaps used in the previous experiments, a series of test-tubes were taken in which various haemolytic quantities of these soaps were placed, and 3 c.c. of fresh sheep's serum added to each tube; the serum and soap were then incubated together for half an hour at 57° C., after which the sheep's corpuscles were added and the tubes replaced in the thermostat; no haemolysis occurred in any of the tubes, even though

1 c.c. 0·01 M of each soap was used, which amount alone would in the case of sodium oleate and linoleate have laked an equal quantity of sheep's red blood corpuscles almost instantaneously, and eight minutes would have sufficed for sodium erucate.

Further observations showed that 0·5 c.c. sheep's serum will exactly protect 1 c.c. 5 per cent. emulsion of sheep's red blood corpuscles against 0·35 c.c. of 0·01 M sodium linoleate. Sheep's serum will also protect its own corpuscles against the natural haemolytic action of pig's serum; for example, we found that 1 c.c. of 5 per cent. emulsion of sheep's red blood corpuscles is completely haemolysed by 0·5 c.c. fresh pig's serum within an hour, the addition, however, of 2 c.c. of sheep's serum will completely inhibit this action.

Cholesterin also has an anti-haemolytic action, though not very marked, 1 c.c. 0·002 M cholesterin emulsion being able to inhibit the action of 0·7 c.c. 0·001 M sodium oleate. Difficulty was experienced in obtaining the cholesterin in a suitable medium to work with, as the solvents of this compound, such as acetone, etc., are mostly haemolytic. In these experiments, therefore, an emulsion of finely suspended cholesterin in normal saline was used, its strength being approximately 0·01 M.

This protective action of serum and cholesterin will be again referred to later on, when we shall have pointed out an action which Sachs and Altmann first described in the case of sodium oleate, and which they termed the behaviour of sodium oleate as anti-complement.

Experiments are described below showing not only that this action can be extended to other unsaturated soaps, but also that it is, to a certain extent, independent of the presence of either complement or amboceptor, and therefore the term 'anti-complement' has been omitted and the word 'balancing' used in its place, as more accurately describing the action. For not only is the haemolytic property of the pig's serum on sheep's red blood corpuscles gradually inhibited as the amount of soap increases, but also after this action has been completely balanced, the serum on its own part further inhibits the haemolytic action of the soap.

Pig's serum is naturally haemolytic for sheep's red blood corpuscles, but if to pig's serum is added a certain quantity of sodium oleate there is an inhibition of haemolysis, and a point can be found where, owing to interaction between these two substances, no haemolysis occurs, although the quantities used of each haemolytic agent are such that if either was used separately complete laking of the sheep's red blood corpuscles would ensue.

For instance, tubes containing:—

a. 1 c.c. sheep's red blood corpuscles—0·5 c.c. pig's serum—4 c.c. normal saline gives complete haemolysis within half an hour.

b. 1 c.c. sheep's red blood corpuscles—0·7 c.c. $\frac{M}{100}$ sodium oleate—3·8 c.c. normal saline results in complete haemolysis in 3 minutes.

But:—

c. 1 c.c. sheep's red blood corpuscles—0·7 c.c. $\frac{M}{100}$ sodium oleate—0·5 c.c. pig's serum —3·3 c.c. normal saline results in almost complete inhibition of haemolysis.

The same holds good for sodium linoleate, and sodium erucate, though the quantities vary in each case.

The results of an experiment are shown graphically in fig. 1. Many similar experiments were carried out giving parallel results:—

In all cases the sodium salt of the acid, the serum and saline were incubated together for three-quarters of an hour at 37° C. prior to adding the sheep's red blood corpuscles. When the contents of the tubes were completed they were again placed in the thermostat at 37° C., and observations made from time to time.

The slight variations in these results are probably due to variations in the 'titer' of the different supplies of pig's serum that were used, as the solutions of oleate and linoleate of soda were the same in each experiment, and, moreover, it may be mentioned that the difference between the balancing points for the two unsaturated soaps is *constant* in all experiments, in each the amount of 0·01 M sodium linoleate required to balance 0·5 c.c. pig's serum being *0·3 c.c. in excess* of the quantity of sodium oleate required for the same result.

It is interesting to note that although according to the previous experiments sodium linoleate alone is a stronger haemolytic agent than sodium oleate, yet it does not seem to be as powerful as the oleate in balancing the action of pig's serum.

These experiments, while showing the balancing action of sodium oleate and linoleate, seemed also to point to the existence of another inhibitory action which might be independent of the existence of complement in the pig's serum.

Some pig's serum was therefore inactivated by heating it at 56° C. for half an hour, and after making sure that the serum was completely inactivated, exactly similar experiments were carried out as before. It was then found that the haemolytic action of the sodium linoleate was inhibited up to the same point when using the inactivated as

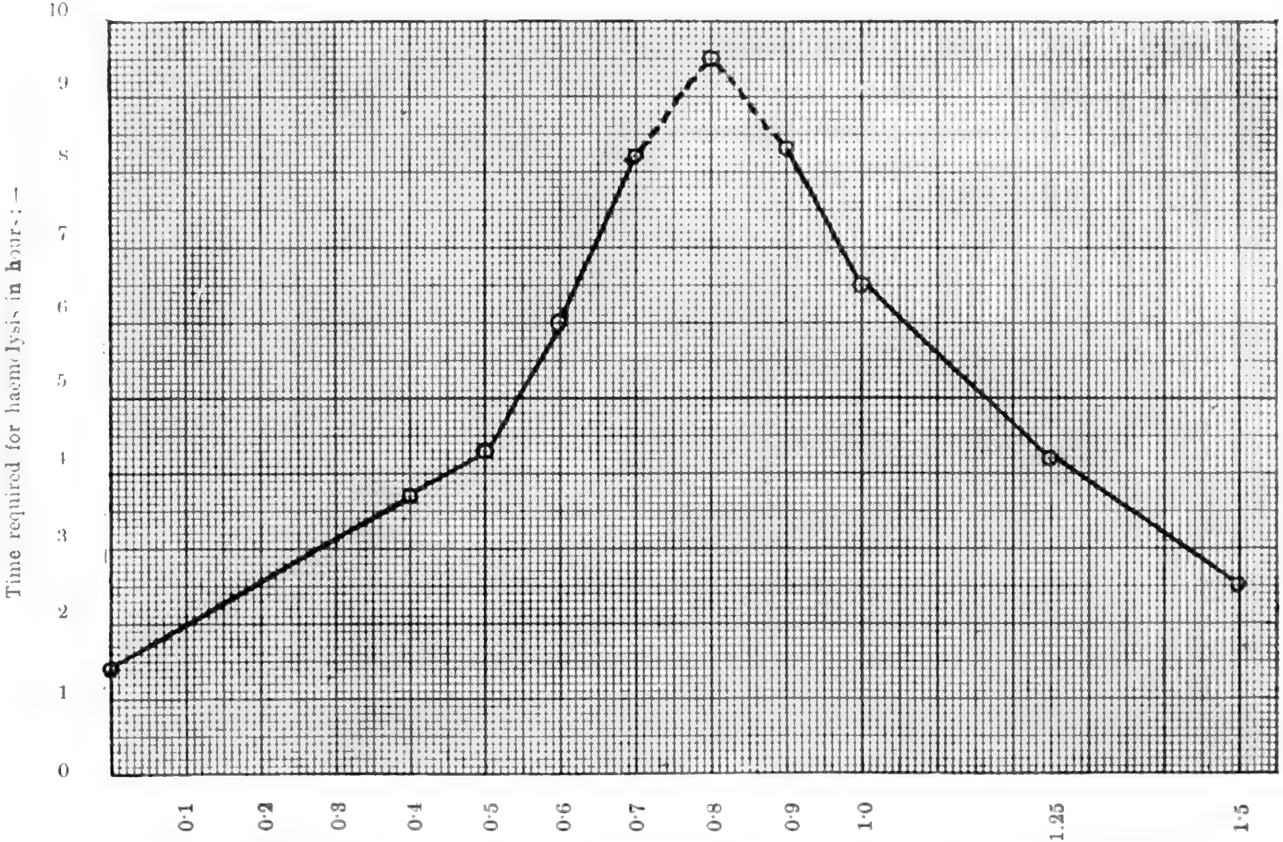

FIG. 1. BALANCING ACTION OF SODIUM LINOLEATE IN PIG'S SERUM

Mixture employed in each case contained 1 c.c. sheep's r. b. c. + 0·5 c.c. pig's serum + amounts in $\frac{1}{10}$ c.c., as indicated above, of $\frac{M}{100}$ sodium linoleate + sufficient normal saline to maintain a constant volume of 5 c.c.

The above chart demonstrates the action of the pig's serum and sodium linoleate in balancing their respective haemolytic actions. The dotted line shows where they almost balance.

when using the fresh serum; that is to say, whereas 1 c.c. 0·01 M sodium linoleate will alone haemolyse 1 c.c. of 5 per cent. emulsion of sheep's red blood corpuscles almost instantaneously, yet when used with either 0·5 c.c. of the fresh or the inactivated pig's serum its action was almost completely inhibited. As a further step, some pig's serum was taken in which both the complement and the amboceptor had been removed. This was accomplished by heating some pig's serum to 55° C. for half an hour to destroy the complement, and then adding excess of sheep's red blood corpuscles (fresh pig's serum being powerfully haemolytic for sheep's red blood corpuscles), incubating the mixture together for an hour at 37° C. and then centrifuging; the clear supernatant serum being again treated with sheep's red blood corpuscles until by tests it was evident that all the amboceptor had combined with the corpuscles, and the clear serum contained neither of its two haemolytic factors.

The effect of this serum on the haemolytic properties of sodium linoleate was then tested in an exactly similar way to that used in the immediately preceding experiments, and it was found that even thus depleted it maintained its protective power intact, and, moreover, that this factor was not in any way diminished. With these results before us, it will be at once apparent that although in the fresh state pig's serum is a powerful haemolyser of sheep's corpuscles, yet if we remove from this serum one or both of its haemolytic factors it then exerts a powerful protective action on the red blood corpuscles, an action which in the case of the sheep's red blood corpuscles is more potent than that exercised by the animal's own serum, for it will be observed that 0·5 c.c. of sheep's serum was able to protect 1 c.c. of a 5 per cent. emulsion of sheep's red blood corpuscles against the haemolytic action of 0·35 c.c. 0·01 M sodium linoleate, while under similar conditions 0·5 c.c. of inactivated pig's serum was able to completely inhibit the haemolytic action of 0·6 c.c. 0·01 M sodium linoleate on a similar quantity of sheep's red blood corpuscles.

We have already mentioned that cholesterin has the power of inhibiting, up to a certain point, the haemolytic action of various soaps.

The question arises, is the above protective action due, as Iscovesco thinks, to cholesterin?

An attempt to investigate this by trying to extract this inhibitory body from pig's serum by means of ether gave a negative reply to this question.

To carry out this experiment 100 c.c. of fresh pig's serum was poured into a separating funnel and 150 c.c. of ether added, the contents being then shaken up together for fifteen minutes, allowed to stand, and then the

serum which collected in the lower part of the funnel was withdrawn. This process was repeated three times, using fresh ether each time. The three portions of ether were then collected into one flask and the ether slowly distilled off at a temperature of 37° C., the last traces being removed by means of a suction pump. The residue containing ether extractives was then shaken up with 30 c.c. of warm normal saline, forming thereby a white opalescent soapy emulsion.

The action of this emulsion was tried on some sheep's red blood corpuscles, but it was found neither to have any haemolytic action alone nor any inhibitory action against other laking agents.

The serum, which had been carefully separated from the ether, was then placed in a flask and all trace of ether removed by bubbling air through, it was then tested with sheep's red blood corpuscles, and it was found that although now it had no haemolytic power, yet its protective action was intact.

The result would appear to be evidence indicating that although cholesterin undoubtedly has considerable inhibitory power, yet it does not account for the protective action of the serum, as the extraction with ether would probably have removed the greater portion.

Additive Effect on Haemolysis of Two Closely Allied Haemolysers which cannot therefore Combine with Each Other

The preceding experiments show that two dissimilar haemolysers, such as the haemolytic substance of pig's serum for sheep's corpuscles and sodium oleate or linoleate, so far from supplementing, balance each other.

The present experiment demonstrates that sodium oleate and sodium linoleate used in common solution aid each other, the effect being approximately additive.

Thus, using the same technique as previously described:—

I. Sodium oleate, 1·25 c.c. of 0·001 M + sheep's r. b. c. 1 c.c. of 5 per cent. emulsion + normal saline to 5 c.c. Result—complete laking in *26 minutes.*

II. Sodium linoleate, 1 c.c. of 0·001 M + sheep's r. b. c. 1 c.c. of 5 per cent. emulsion + normal saline to 5 c.c. Result—complete laking in *19 minutes.*

III. The same quantities of the two together, viz., sodium oleate 1·25 c.c. of 0·001 M + sodium linoleate 1 c.c. of 0·001 M + sheep's r. b. c. 1 c.c. of 5 per cent. emulsion + saline to 5 c.c. Result—complete laking in *11 minutes.*

IV. Half the initial amounts of the two sodium salts gave the following results, viz.:— Sodium oleate, 0·62 c.c. of 0·001 M + sodium linoleate 0·5 c.c. of 0·001 M + sheep's r. b. c. 1 c.c. of 5 per cent. emulsion + saline to 5 c.c. Result—laking in *28 minutes.*

There is accordingly no balancing here, and the effects of the two used in common solution are practically a purely additive function.

When sodium linoleate and fresh lecithin are used as a pair of haemolysers, a very distinct balancing action is obtained. Thus, 0·2 c.c. of 0·01 M sodium linoleate laked 1 c.c. of 5 per cent. emulsion of sheep's red blood corpuscles in four minutes; but when exactly the same amount of the sodium linoleate is first treated with 0·5 c.c. of an emulsion corresponding to 0·01 M lecithin, the haemolysis is delayed for about two and three-quarter hours.

This illustrates very clearly the constituent in the red blood corpuscle which is attacked in haemolysis, and also shows that the haemolyser and the lecithin of the corpuscles enter into at least a quasi-combination, so limiting the amount of corpuscle haemolysable by a given amount of haemolyser.

Relationships of Oxidizing and Reducing Agents to Haemolysis

Hydrogen Peroxide and Haemolysis.—At the outset of this investigation an attempt was made to test whether complement could be replaced by hydrogen peroxide.

Pig's serum, which, as is well known, has a haemolytic action on sheep's red blood corpuscles, was inactivated, and to different dilutions of serum varying strengths of hydrogen peroxide were added. Repeated experiments yielded discordant results; a strength of hydrogen peroxide which on one occasion caused haemolysis, failed to do so a second time.

Investigation showed that the hydrogen peroxide had an acid reaction, and this was probably the cause of the uncertain results.

On using dilutions of Merck's perhydrol, much more uniform results were obtained. Except in very strong solutions up to 1 in 50 in normal saline—perhydrol had no haemolytic action on fresh sheep's red corpuscles. Added to inactivated pig's serum it had no complementary action, and, indeed, the haemolytic action of the stronger solutions seemed to be inhibited by the serum.

Another oxidizing agent, quinone, was tried in varying strengths in a similar way. No haemolytic or complementary action was obtained.

Added to *fresh* pig's serum, neither perhydrol nor quinone interfered with its haemolytic power.

Similarly, oxidase containing solutions from fresh vegetable juices could not be used as substitutes for complement.

Thus, we have been unable to trace the nature and action of complement in haemolysis, but our experiments led us incidentally to certain observations upon the effects of reducing and oxidizing substances on haemolysis which are here recorded.

Effect of Reducing Agents on Complement

We next tried the effect of various dilutions of ammonium sulphide on the haemolytic action of pig's and other sera. The stock ammonium sulphide used for dilutions was of a strength 0·4 M. Table I gives the details of the experiments. The results show that in dilution up to 1 in 1,000 the ammonium sulphide inhibits the haemolytic action of the sera used.

Equivalent solutions of hydrogen sulphide and ammonia in normal saline have not such inhibitory action.

In all experiments the tubes were all made up to 5 c.c. with normal saline.

The sheep's red corpuscles were used in the form of a 5 per cent. suspension in normal saline.

Fresh guinea-pig's serum was used as complement.

The anti-sheep haemolytic rabbit's serum was of such a titre that 1 c.c. of a 1 in 1,500 dilution dissolved 1 c.c. of a 5 per cent. suspension of sheep's red blood corpuscles in half an hour.

Table I

1.	1 c.c. of fresh pig's serum + 1 c.c. ammonium sulphide	(dilution 1 in 50)	+ 1 c.c. r. b. c.—	No haemolysis
2.	,, ,,	(dilution 1 in 100)	,,	,,
3.	,, ,,	(dilution 1 in 200)	,,	,,
4.	,, ,,	(dilution 1 in 300)	,,	Partial haemolysis
5.	1 c.c. of inactivated serum + 0·1 c.c. complement + 1 c.c. ammonium sulphide	(dilution 1 in 50)	+ 1 c.c. r. b. c.—	No haemolysis
6.	,, ,,	(dilution 1 in 100)	,,	,,
7.	,, ,,	(dilution 1 in 200)	,,	Haemolysis
8.	,, ,,	(dilution 1 in 300)	,,	,,
9.	1 c.c. inactivated rabbit's serum (dilution 1 in 20) + 0·1 c.c. complement + 1 c.c. ammonium sulphide	(dilution 1 in 50)	+ 1 c.c. r. b. c.—	No haemolysis
10.	,, ,,	(dilution 1 in 100)	,,	,,
11.	,, ,,	(dilution 1 in 200)	,,	Slight haemolysis
12.	,, ,,	(dilution 1 in 300)	,,	Complete haemolysis
13.	1 c.c. fresh pig's serum + 1 c.c. r. b. c.			Haemolysis
14.	1 c.c. inactivated pig's serum + 0·1 c.c. complement + 1 c.c. r. b. c.			,,
15.	1 c.c. inactivated rabbit's serum + 0·1 c.c. complement + 1 c.c. r. b. c.			,,
16.	1 c.c. inactivated pig's serum + 1 c.c. r. b. c.			No haemolysis
17.	1 c.c. inactivated rabbit's serum + 1 c.c. r. b. c.			,,
18.	1 c.c. ammonium sulphide (dilution 1 in 50) + 1 c.c. r. b. c.			,,

All tubes were made up to 5 c.c. with normal saline.

The sera and ammonium sulphide were incubated together at 37° C. for half an hour, r.b.c. emulsion then added, and whole incubated for one hour.

A comparison of the effects of equivalent strengths of sodium hydro-oxide, ammonium hydrate, and ammonium sulphide on sheep's r.b.c. are given by Table II..

TABLE II

1. 0·5 c.c. $\frac{N}{10}$ NaOH + 1 c.c. r. b. c. — Haemolysis in half an hour
2. 0·5 c.c. $\frac{N}{10}$ NH_4OH + 1 c.c. r. b. c. — Slight haemolysis next day
3. 0·5 c.c. $\frac{N}{10}$ $(NH_4)_2S$ + 1 c.c. r. b. c. — No haemolysis

All tubes made up to 5 c.c. with normal saline.

In Tables III, IV and V are shown the 'balancing' action of sodium hydrate with fresh and inactivated serum; also the influence of perhydrol on this action. All tubes were made up to 5 c.c. with normal saline, incubated at 37° C. for two hours, and results noted.

TABLE III

1.	1 c.c. fresh pig's serum + 1 c.c. r. b. c.	Haemolysis
2.	„ „ + 0·5 c.c. $\frac{N}{10}$ NaOH	No haemolysis
3.	„ „ + 0·5 c.c. $\frac{N}{15}$ NaOH	„
4.	„ „ + 0·5 c.c. $\frac{N}{20}$ NaOH	Haemolysis
5.	„ „ + 0·5 c.c. $\frac{N}{25}$ NaOH	„
6.	„ „ + 0·5 c.c. $\frac{N}{30}$ NaOH	„
7.	0·5 c.c. $\frac{N}{10}$ NaOH + 1 c.c. r. b. c.	„
8.	0·5 c.c. $\frac{N}{15}$ NaOH + 1 c.c. r. b. c.	„
9.	0·5 c.c. $\frac{N}{20}$ NaOH + 1 c.c. r. b. c.	„
10.	0·5 c.c. $\frac{N}{25}$ NaOH + 1 c.c. r. b. c.	No haemolysis
11.	0·5 c.c. $\frac{N}{30}$ NaOH + 1 c.c. r. b. c.	„

TABLE IV

1.	1 c.c. fresh pig's serum + 1 c.c. r. b. c.	Haemolysis
2.	0·5 c.c. $\frac{N}{10}$ NaOH + 1 c.c. r. b. c.	„
3.	1 c.c. fresh pig's serum + 0·5 c.c. $\frac{N}{10}$ NaOH + 1 c.c. r. b. c. ...	No haemolysis
4.	„ „ „ + 1 c.c. perhydrol (1 in 30 dilution) ...	Haemolysis
5.	„ „ „ + 1 c.c. perhydrol (1 in 60 dilution)	„
6.	1 c.c. perhydrol (1 in 30 dilution) + 1 c.c. r. b. c.	No haemolysis
7.	„ (1 in 60 dilution) + 1 c.c. r. b. c.	„

TABLE V

1	1 c.c. inactivated pig's serum + 0·5 c.c. $\frac{N}{10}$ NaOH + 1 c.c. r. b. c. ...	Slight haemolysis in 4 hours
2.	„ „ „ + 1 c.c. perhydrol (1 in 30 dilution)	Haemolysis in 2 hours
3	„ „ „ + 1 c.c. perhydrol (1 in 60 dilution)	Haemolysis in 2 hours
4.	„ + 1 c.c. r. b. c. + 1 c.c. perhydrol (1 in 30 dilution)	No haemolysis
5.	„ „ „ + 1 c.c. perhydrol (1 in 60 dilution)	No haemolysis

The haemolytic action of ammonia in various strengths on sheep's r.b.c. is shown in Table VI.

TABLE VI

1.	3 c.c. $\frac{N}{10}$ ammonia + 1 c.c. r. b. c.	Haemolysis in 2 or 3 minutes
2.	2 c.c. „ „	„ „
3.	1·5 c.c. „ „	Partial haemolysis in 2 hours
4.	1 c.c. „ „	Slight haemolysis in 2 hours
5.	0·75 c.c. „ „	No haemolysis in 2 hours
6.	0·5 c.c. „ „	„ „

All tubes were made up to 5 c.c. with normal saline.

Table VII gives the effects of adding ammonia to fresh and inactivated pig's serum, and of variations in the technique.

TABLE VII

1.	1 c.c. $\frac{N}{10}$ ammonia + 1 c.c. fresh pig's serum	Slight haemolysis next day
2.	0·75 c.c. „ „	Very slight haemolysis next day
3.	0·5 c.c. „ „	„ „
4.	1 c.c. $\frac{N}{10}$ ammonia + 1 c.c. inactivated pig's serum	Pale brown colour next day
5.	0·75 c.c. „ „ ...	„ „
6.	0·5 c.c. „ „ ...	„ „

Tubes made up to 4 c.c. with normal saline, incubated for one hour at 37° C., then 1 c.c. r.b.c. added to each tube and incubated for two hours.

7.	1 c.c. $\frac{N}{10}$ ammonia + 1 c.c. fresh pig's serum + 1 c.c. r. b. c.	Slight haemolysis next day
8.	0·75 c.c. „ „ ...	Very slight haemolysis next day
9.	0·5 c.c. „ „ ...	„ „
10.	1 c.c. $\frac{N}{10}$ ammonia + 1 c.c. inactivated pig's serum + 1 c.c. r. b. c.	Pale brown colour
11.	0·75 c.c. „ „ „	„ „
12.	0·5 c.c. „ „ „	„ „

Tubes made up to 5 c.c. with normal saline and r.b.c. at once, and incubated for two hours.

Having found that quinone alone did not much influence haemolysis, we next tried the effect of quinone with ammonia added. The alkali promptly turns the quinone black, or brown in weak dilutions, probably turning it into hydroquinone, which then undergoes some further change. Quinone added to a dilution of ammonia, which by itself had no haemolytic action in two hours, promptly turned black and produced instant haemolysis. Inactivated pig's serum inhibits this action to some extent. Hydroquinone alone has no haemolytic effect, but with ammonia produces the same results as quinone. The results obtained are set forth in **Table VIII.**

Table VIII

1.	**0·5 c.c.** $\frac{N}{10}$ **ammonia** + 1 c.c. r. b. c.	**No haemolysis**
2.	,, ,, ,, + 1 c.c. 0·1 % quinone	Dark brown colou Haemolysis in 2
3.	**0·2 c.c.** ,, ,, ,, ,, ...	,,
4.	**0·1 c.c.** ,, ,, ,, ,, ...	Brown colour No haemolysis in
5.	**0·5 c.c.** ,, ,, ,, ,, + 1 c.c. inactivated pig's serum ...	Black colour No haemolysis
6.	**1 c.c. 0·1 % quinone** + 1 c.c. r. b. c.	,, ,,
7.	**1 c.c. 0·1 % hydroquinone** + 1 c.c. r. b. c.	,, ,,
8.	,, ,, ,, ,, ,, + 1 c.c. inactivated pig's serum	,, ,,
9.	**0·5 c.c.** $\frac{N}{10}$ **ammonia** + 1 c.c. r. b. c. + 1 c.c. 0·1 % hydroquinone	**Black colour** **Haemolysis**
10.	,, ,, ,, ,, ,, + 1 c.c. inactivated pig's serum	**Brown colour** **No haemolysis**
11.	**0·1 c.c.** $\frac{N}{10}$ **ammonia** + 1 c.c. r. b. c. + 1 c.c. 0·1 % hydroquinone	Dark brown colo No haemolysis
12.	,, ,, ,, ,, ,, + 1 c.c. inactivated pig's serum	**Brown colour** **No haemolysis**

All tubes made up to 5 c.c. with normal saline.

Summary of Results

1. The substances concerned in haemolysis, including thereby both the haemolytic agent outside and the substance attacked within, have a powerful mutual effect upon one another's solubilities.

2 Instances are given of such increased solubilities, and the favouring effects upon haemolysis noted. As a result of such increased solubility lecithides are dissolved out from the mass of the corpuscle, so setting free the haemoglobin also, and laking is the result.

3. It is noted that all the haemolytic class of unsaturated soaps of

fatty acids, saponin, mowrin, digitalin, the various bile salts, possess common physical, chemical and physiological properties, and are all unsaturated bodies capable of bromination, etc.

4. The similar action upon the heart of the haemolytic bodies is probably due to combination between these and the heart lipoids.

5. Although want of saturation exists, it is probable that the first fundamental step is a 'physical' one of lowering of surface tension with accompanying tendency to solution. But no hard and fast line can be drawn between so-called physical and chemical action.

6. The balancing action of haemolysers is discussed, and it is shown that this is not obtained with closely similar haemolysers, where instead an additive action is seen. This suggests that balancing is due to a combination or interlocking of the two haemolysers, whereby nothing is left free to touch the corpuscles.

7. Where sera and such haemolysers as sodium oleate balance, the first call is between the active body of the serum and the oleate; next, in absence of the active body or of complement, the serum proteins alone, although not active in themselves as haemolysers, possess a superior binding power over the corpuscles for the oleate, and hence act as protectors, so that much more oleate in excess must be added before the corpuscles are attacked. Accordingly, as is well known, a mere trace of oleate suffices to break down corpuscles in saline suspension, but in serum suspension many times more oleate must be added before any result is obtained.

8. It follows from this that oleates, etc., do not act as anti-complements, and ought not to be described as such; it is most probable that they possess no specific relationship whatever to complement.

9. Sodium oleate can also be balanced by lecithin for similar reasons.

10. Under conditions specified, and in alkaline solution, oxidizing agents favour haemolysis, and reducing agents restrain it; but an oxidizing agent alone cannot replace complement in an active haemolytic serum, and it is not probable that complement has the nature of a peroxide body.

OBSERVATIONS ON THE HAEMOLYTIC ACTION OF CERTAIN BILE DERIVATIVES

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Our knowledge of the processes involved in the laking of red blood corpuscles is at present in an obscure and unsatisfactory condition, and despite the amount of research carried out on haemolysis and the number of theories advanced to account for different results, many cases still remain unexplained.

In connection with this subject, one very interesting point has lately been advanced by St. Faust and Tallqvist, and afterwards by Moore: these observers found that haemolytic action, when brought about by such bodies as the fatty acids, occurred only in cases where the acid employed was one of an unsaturated variety containing in its molecule one or more double bonds, while the corresponding saturated bodies produced no effect. In view of this observation, it is likely that the state of combination of the carbon atom constitutes an important factor in many cases of haemolysis produced by chemical agents.

The strong probability, on chemical grounds, of certain bile products being constituted by bodies of an unsaturated nature suggested the present investigation: the curious results obtained do not seem capable of explanation by any of the theories put forward at present.

Substances used

The present paper deals with the results of haemolysis by three bile derivatives the sodium salt of glycocholic acid, cholalic acid and choleic acid. These were prepared as follows:

Sodium Glycocholate

Fresh ox bile was freed from pseudo-mucin by treatment with alcohol: after the evaporation of the alcohol, neutral acetate of lead was added, the resulting precipitate separated off and decomposed by heat in

the presence of a sodium carbonate solution. The mixture was evaporated to dryness and the residue extracted with alcohol; the alcoholic extract was filtered, the filtrate evaporated to dryness and the residue dissolved in water. This watery solution of sodium glycocholate was now decolorised by animal charcoal, and the free glycocholic acid thrown out by means of a dilute solution of hydrochloric acid. The acid was thoroughly washed with water, then dried and again dissolved in alcohol. To this alcoholic solution sodium carbonate was added, and the whole again evaporated to dryness. Residue was dissolved in a little cold alcohol, and the mixture filtered. This process of purification by dissolving in alcohol was repeated; the alcoholic solution was then evaporated to dryness and the residue dissolved in a little water, filtered, evaporated to dryness over the water bath, and ultimately dissolved in normal saline solution, and in this solution utilised for the experiments.

Cholalic Acid

Ox bile was boiled for thirty hours with one-fifth of its volume of 30 per cent. caustic soda, the total volume of the mixture being kept constant by the addition of water from time to time. The solution was then saturated with carbon dioxide and evaporated almost to dryness. Residue was extracted with 96 per cent. alcohol, and the extract diluted with water so as to contain not more than about 20 per cent. of alcohol; it was then treated with a solution of barium chloride, the precipitate filtered off, the filtrate treated with weak hydrochloric acid, and the precipitated cholalic acid separated off and thoroughly washed with water. This acid was now changed into the sodium salt, and the above process of treatment with barium chloride, etc., repeated twice. The free acid ultimately obtained was dissolved in alcohol, and by the addition of sodium carbonate again changed into the sodium salt; the alcoholic solution was filtered, evaporated to dryness, the residue dissolved in a little cold alcohol and filtered; filtrate was evaporated to dryness, residue dissolved in water, filtered, again evaporated to dryness and final residue utilised for experiments.

Choleic Acid

Choleic acid was prepared from the precipitate obtained by barium chloride in the preparation of cholalic acid: this precipitate, which consisted of an impure salt of barium choleate, was purified on the lines described above; it was also utilised in the form of the sodium salt.

Experiments

The first set of experiments was carried out with the sodium salt of cholalic acid. In all cases red blood corpuscles from the sheep were used; these were carefully washed four times in the ordinary way with normal salt solution, and then made into a 5 per cent. emulsion with isotonic saline.

For convenience in preparing the somewhat varied strengths of cholalic acid utilised in the experiments, two solutions were made—one a deci-molecular and the other a centi-molecular; in each case the molecular weight of the salt was taken as corresponding to the formula $C_{24}H_{39}O_5Na$, all solutions being made in normal saline.

For each experiment a series of tubes, each containing 1 c.c. of the above emulsion of sheep's red blood corpuscles, was taken, and to this was added a measured amount of sodium cholalate. By the addition of the necessary amount of normal saline, the final volume of each tube was maintained constant, and in all our experiments amounted to 5 c.c. The tubes were then placed in the incubator at 37° C., and observations constantly taken at short intervals.

As the result of repeated experiments, it was found that this salt displayed well-marked haemolytic properties, but at the same time exhibited some peculiarities so striking as to amount to what may be suggestively termed a haemolytic paradox.

In tubes containing a moderately strong dose of sodium cholalate, the corpuscles were completely laked in intervals of from one to two hours; when the substance was present in somewhat smaller quantities, haemolysis was delayed for three or four hours: on still further reduction of the amount of substance, however, the peculiar fact was observed that haemolysis took place in a shorter and shorter space of time, until a point was reached at which a minimum dose gave a maximum effect. With such an amount this *minimal-optimum* dose often caused complete laking of the corpuscles in six minutes, or even less; while on the other hand, a dose several times as great might take hours to produce a similar effect.

When exhibited in amounts smaller than this minimal-optimum dose, the effect became gradually less, until ultimately a strength was used beyond which no further haemolysis could be obtained.

The accompanying chart gives a good idea of the results obtained; in a long series of experiments the laking always took place as indicated.

Chart No. I

Sodium Cholalate

Time during which sodium cholalate had been acting				Minutes: 5	13	20	30	40	53	65	80	115	155	320
1 c.c. sheep's r. b. c. +			+ 4 c.c. $\frac{M}{5}$ sodium cholalate	–	✦	✦	✦	✦	✦	✦	✦	✦	✦	✦
,, ,,	+ 1 c.c.	saline......	+ 3 ,, ,,	–	–	–	✦	✦	✦	✦	✦	✦	✦	✦
,, ,,	+ 1 ,,	,,	+ 3 c.c $\frac{M}{10}$ sodium cholalate	–	–	–	–	–	–	–	✦	✦	✦	✦
,, ,,	+ 2 ,,	,,	+ 2 ,, ,,	–	–	–	–	–	–	–	–	✦	✦	✦
,, ,,	+ 3 ,,	,,	+ 1 ,, ,,	–	–	–	–	–	–	–	–	–	✦	✦
,, ,,	+ 3·1 ,,	,,	+ 0·9 ,, ,,	–	–	–	–	–	–	–	–	–	✦	✦
,, ,,	+ 3·2 ,,	,,	+ 0·8 ,, ,,	–	–	–	–	–	–	–	–	–	–	✦
,, ,,	+ 3·3 ,,	,,	+ 0·7 ,, ,,	–	–	–	–	–	–	–	–	–	✦	✦
,, ,,	+ 3·4 ,,	,,	+ 0·6 ,, ,,	–	–	–	–	–	–	–	–	✦	✦	✦
,, ,,	+ 3·5 ,,	,,	+ 0·5 ,, ,,	–	–	–	–	–	–	–	–	✦	✦	✦
,, ,,	+ 3·6 ,,	,,	+ 0·4 ,, ,,	–	–	–	–	–	–	✦	✦	✦	✦	✦
,, ,,	+ 3·7 ,,	,,	+ 0·3 ,, ,,	–	–	–	–	–	✦	✦	✦	✦	✦	✦
,, ,,	+ 2 ,,	,,	+ 0·2 ,, ,,	–	–	✦	✦	✦	✦	✦	✦	✦	✦	✦
,, ,,	+ 3 ,,	,,	+ 0·1 ,, ,,	✦	✦	✦	✦	✦	✦	✦	✦	✦	✦	✦
,, ,,	+ 3·5 ,,	,,	+ 0·05 ,, ,,	–	–	–	–	✦	✦	✦	✦	✦	✦	✦
,, ,,	+ 3·6 ,,	,,	+ 0·04 ,, ,,	–	–	–	–	–	–	✦	✦	✦	✦	✦
,, ,,	+ 3·7 ,,	,,	+ 0·03 ,, ,,	–	–	–	–	–	–	–	✦	✦	✦	✦
,, ,,	+ 3·8 ,,	,,	+ 0·02 ,, ,,	–	–	–	–	–	–	–	–	–	–	–
,, ,,	+ 3·9 ,,	,,	+ 0·01 ,, ,,	–	–	–	–	–	–	–	–	–	–	–
,, ,,	+ 4 ,,	,,	(control)	–	–	–	–	–	–	–	–	–	–	–

Note.—The emulsion of red corpuscles was rapidly added last of all, the difference in time between the first and last tube being barely 4 minutes. Immediately after the corpuscles were added, the tubes were placed in a thermostat [37° C.] where they were observed.

The times were calculated from the moment when the tubes were placed in the thermostat.

In measuring amounts of sodium cholalate less than 0·2 c.c., an $\frac{M}{100}$ solution was used.

✦ Denotes point at which laking became complete.

The results are also shown graphically plotted in fig. 1, in which the ordinates represent time of complete haemolysis in minutes, and the abscissae, the concentrations of the sodium cholalate, in c.c., as indicated, of 0·1 M solution added to a total volume of 5 c.c. when completely made up.

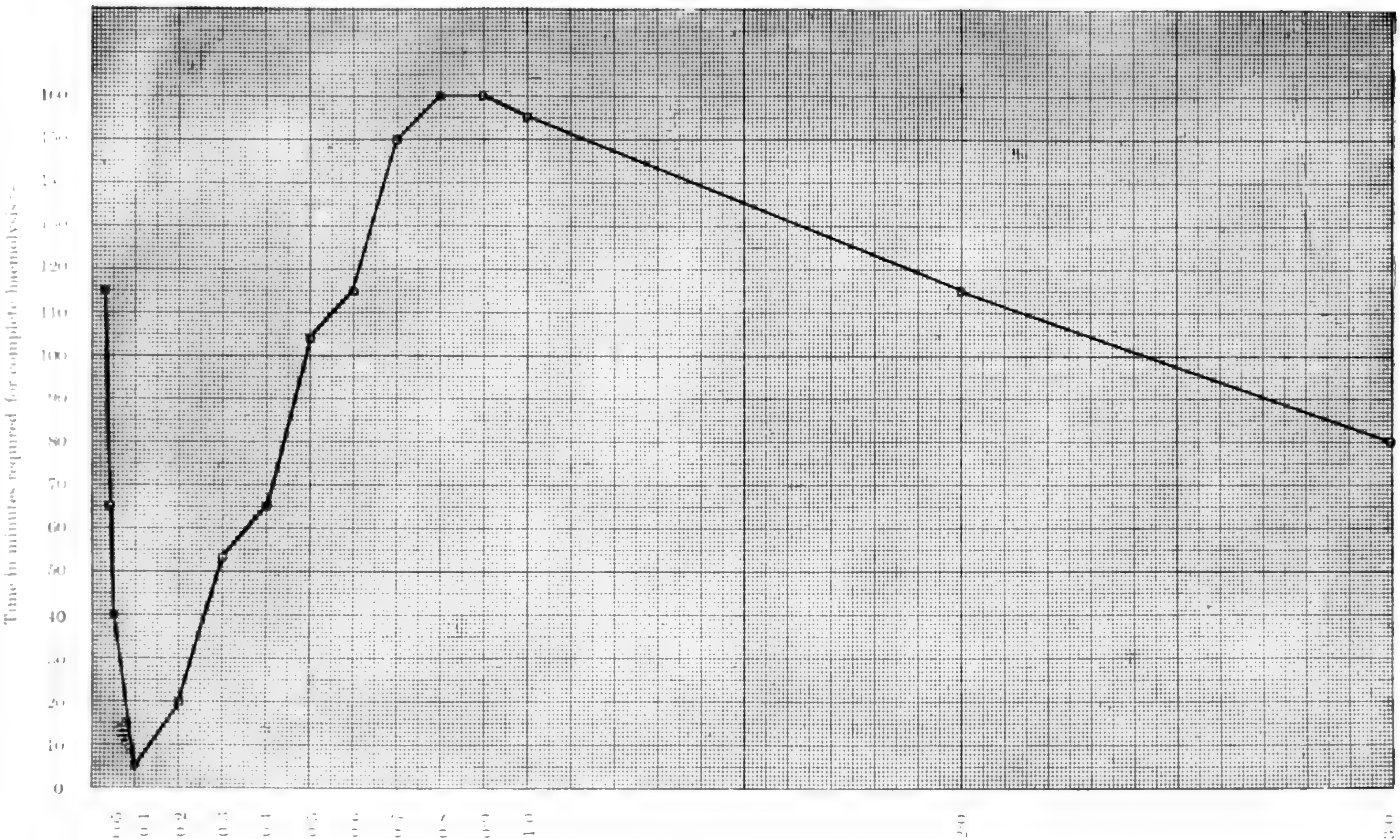
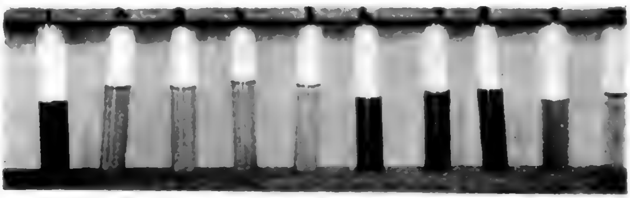


FIG. 1. SODIUM CHOLALATE

Each test tube contained 1 c.c. sheep's r. b. c. (5 % emulsion) + amounts, as indicated above, in cubic centimetres of $\frac{M}{10}$ sodium cholalate + sufficient normal saline to maintain a constant volume of 5 c.c.

The following reproduction also shows these results; the tubes were left to stand for one hour and then centrifuged; by this means a more suitable condition for photographic purposes was obtained. Haemolysis is distinctly seen in tubes 1, 6, 7 and 8, the remainder being quite free from laking, with the exception of Nos. 2 and 9, where a slight action has taken place.

From these results, it is obvious that the haemolytic power of the so called minimal-optimum dose is much more marked than that obtained when much larger amounts are used, and that between these two points there lies a sort of neutral ground where the haemolytic power is very much reduced. In the experiment recorded in the chart it is seen that



1 2 3 4 5 6 7 8 9 10

Photograph showing Haemolytic Paradox

Tubes with black appearance are those in which laking has occurred.

The following table indicates the amount of sodium cholalate in each tube in the photograph:—

No. **1**.	3 c.c. $\frac{M}{5}$	Sodium	cholalate	No. **6**.	0·3 c.c. $\frac{M}{10}$	Sodium	cholalate
,, **2**.	,, $\frac{M}{10}$	,,	,,	,, **7**.	0·2 c.c. ,,	,,	,,
,, **3**.	1 c.c. ,,	,,	,,	,, **8**.	0·5 c.c. $\frac{M}{100}$	,,	,,
,, **4**.	0·8 c.c. ,,	,,	,,	,, **9**.	0·3 c.c. ,,	,,	,,
,, **5**.	0·5 c.c. ,,	,,	,,	,, **10**.	0·2 c.c. ,,	,,	,,

0·1 c.c. of 0·1 **M**. sodium cholalate lakes completely in five minutes, whereas it takes thirteen minutes for 4 c.c. of 0·2 **M** solution to produce the same effect: in other words, a certain minimum dose gives in *five* minutes an effect equivalent to that produced by a dose about *eighty* times as great in *thirteen* minutes.

In another series of experiments performed under the same conditions, but using the sodium salt of choleic acid, similar results were obtained.

When a strength of 4 c.c. 0·1 M sodium choleate was used, haemolysis was complete in about *six* minutes; in this strength laking was quickly followed by an action on the liberated haemoglobin, as indicated by a dark brown coloration of the liquid. With 0·8 c.c. 0·1 M solution the full effect was in evidence in about *eleven* minutes; when, however, only 1 c.c. 0·01 M was used, haemolysis was almost instantaneous, the corpuscles being completely laked within *one* minute.

This salt is a much more powerful agent than the corresponding compound with cholalic acid. Whereas with sodium cholalate a solution of 0·3 c.c. 0·01 M required about eighty minutes for complete laking, and a strength corresponding to 0·2 c.c. 0·01 M gave only the merest indication of a positive reaction after twenty hours' observation, with sodium choleate in strength of 0·7 c.c. 0·001 M complete haemolysis was obtained in one hundred and sixty minutes, and with 0·6 c.c. 0·001 M in twenty hours: even with 0·5 c.c. 0·001 M, traces of haemolysis were evident after this time.

The full results of one experiment are indicated in Chart 2.

Similar experiments, giving in the main the same kind of result, were performed with sodium glycocholate. Here, however, there was not the same marked difference between the haemolytic action of large and small amounts as was seen in the case of the above described salts; this is due to the fact that the action of the minimal-optimum dose is in this case not nearly so rapid. In other respects, as indicated by the chart, the same general result is in evidence. The results are shown also in fig. 3.

On the other hand, haemolysis was ultimately obtained with smaller amounts than in the case of sodium cholalate, though, even in this respect, it did not appear to be so powerful as the choleic salt.

A few observations were also made with free cholalic acid; considerable difficulty was experienced in procuring a suitable method of application owing to its lack of solubility in inert substances. We succeeded, however, in emulsifying a small amount with normal saline, and with this emulsion similar results to those obtained with the sodium compound were noticed; that is to say, with a certain small amount of emulsion a much more marked haemolysis was produced in a given period than was in evidence after the exhibition of perhaps nine or ten times as much in a much longer space of time.

Chart No. II

Sodium Choleate

Time during which sodium choleate had been acting (Minutes)	1	2	3	6	7	8	9	11	20	31	50	105	140	160	240	1200
1 c.c. sheep's r. b. c. + ... + 4 c.c. $\frac{M}{10}$ sodium choleate	–	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 1 c.c. saline + 3	–	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 2 .. .. + 2	–	–	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3 .. .. + 1	–	–	–	–	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·1 .. .. + 0·9	–	–	–	–	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·2 .. .. + 0·8	–	–	–	–	–	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·3 .. .. + 0·7	–	–	–	–	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·4 .. .. + 0·6	–	–	–	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·5 .. .. + 0·5	–	–	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·6 .. .. + 0·4	–	–	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·7 .. .. + 0·3	–	–	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·8 .. .. + 0·2	–	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·9 .. .. + 0·1	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·5 .. .. + 0·75 c.c. $\frac{M}{100}$ ..	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·6 .. .. + 0·4	–	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·7 .. .. + 0·3	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·8 .. .. + 0·2	–	–	–	–	–	–	–	✚	✚	✚	✚	✚	✚	✚	✚	✚
.. .. + 3·0 .. .. + 1·0 c.c. $\frac{M}{1000}$ ..	–	–	–	–	–	–	–	–	–	–	✚	✚	✚	✚	✚	✚
.. .. + 3·2 .. .. + 0·8	–	–	–	–	–	–	–	–	–	–	–	–	✚	✚	✚	✚
.. .. + 3·3 .. .. + 0·7	–	–	–	–	–	–	–	–	–	–	–	–	–	✚	✚	✚
.. .. + 3·4 .. .. + 0·6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	✚
.. .. + 3·5 .. .. + 0·5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	O
.. .. + 3·6 .. .. + 0·4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
.. .. + 3·7 .. .. + 0·3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
.. .. + 4 .. .. (control)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

✚ Indicates complete haemolysis.

Note. The same conditions existed during this experiment as in the preceding Chart except that the tubes were not placed in the thermostat for 8 minutes after adding the corpuscles. The corpuscles were added during $2\frac{1}{2}$ minutes, and the times are calculated from the time when the last tube received its corpuscle.

O Trace of haemolysis.

These results are shown graphically in Fig. 2, where ordinates show as in Fig. 1.

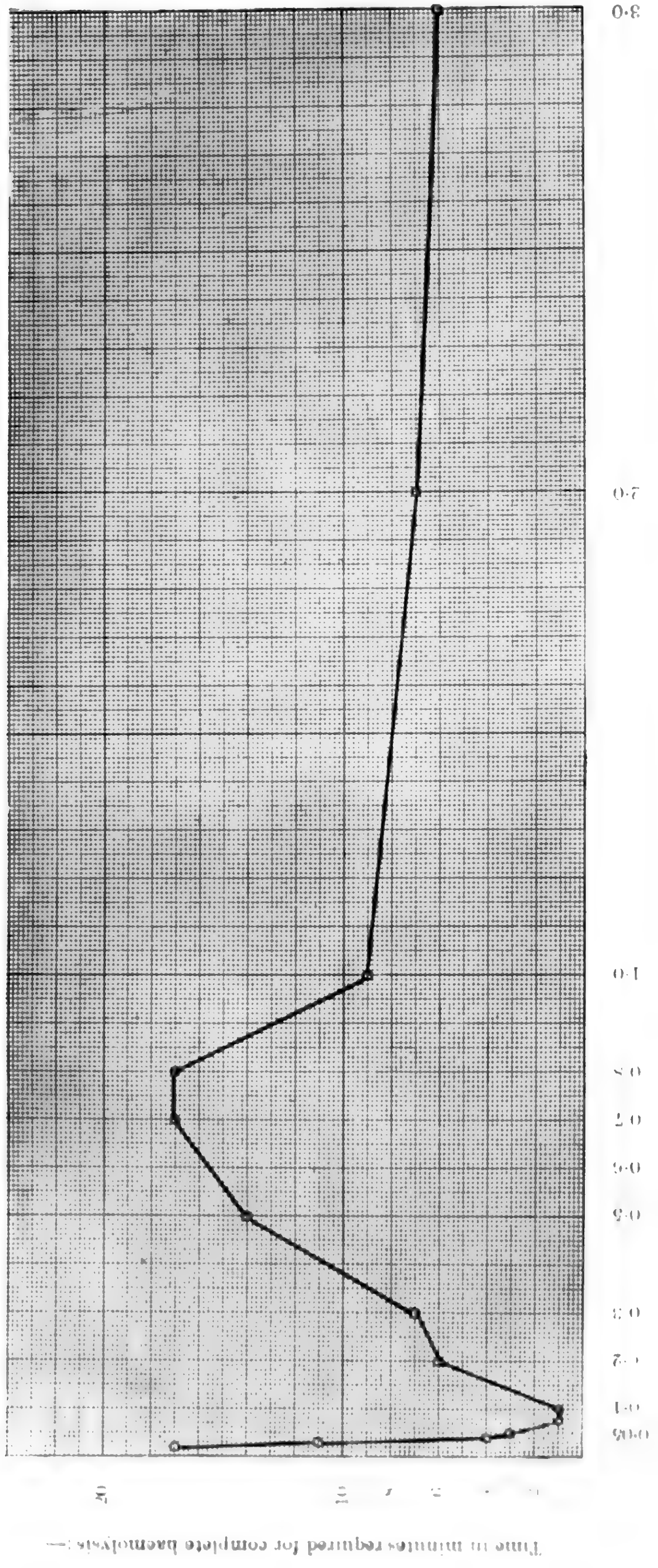


Fig. 2. Sodium Choleate

Each test tube contained 1 c.c. sheep's r. b. c. (5 % emulsion) + amounts in cubic centimetres, as indicated above, of $\frac{M}{10}$ sodium choleate + sufficient normal saline to maintain a constant volume of 5 c.c.

The curve shows well the paradoxical result that a small amount of the haemolyser is more efficient than a larger amount.

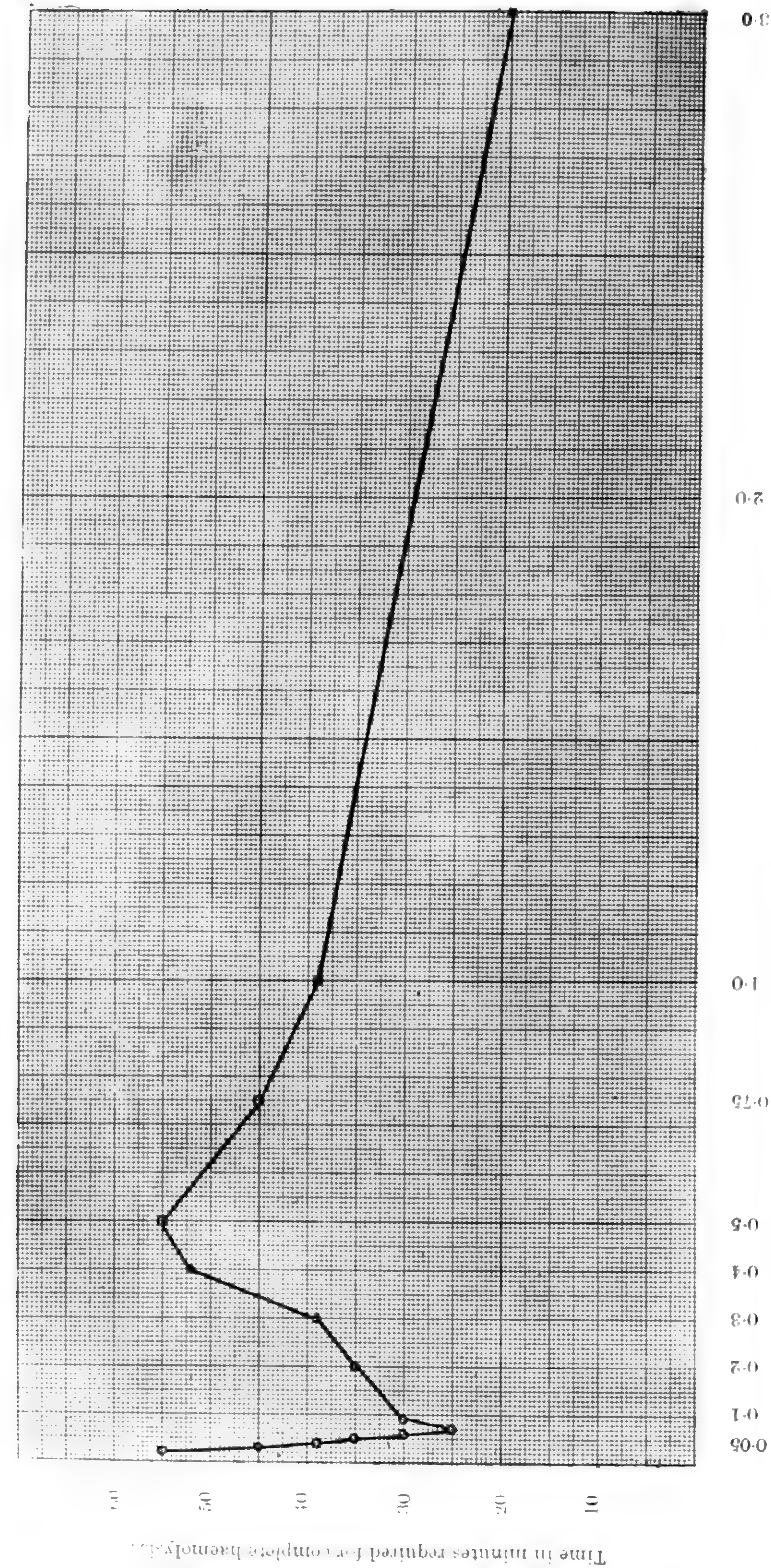


FIG 3. SODIUM GLYCOCHOLATE

Each test-tube contained 1 c.c. sheep's r. b. c. (5% emulsion) + amounts in cubic centimetres, as indicated above, of $\frac{M}{10}$ sodium glycocholate + sufficient normal saline to maintain a constant volume of 5 c.c.

Chart No. III

Sodium Glycocholate

Times in minutes	25	30	35	40	44	50	60	80	20 hours
1 c.c. sheep's r. b. c. + 1 c.c. saline + 3 c.c. $\frac{M}{10}$ sodium glycocholate	✦	✦	✦	✦	✦	✦	✦	✦	✦
" " + 3 " " + 1 " " "	–	–	–	–	✦	✦	✦	✦	✦
" " + 3·25 " " + 0·75 " " "	–	–	–	–	–	✦	✦	✦	✦
" " + 3·5 " " + 0·5 " " "	–	–	–	–	–	–	✦	✦	✦ 1st
" " + 3·6 " " + 0·4 " " "	–	–	–	–	–	–	✦	✦	✦ 2nd
" " + 1 " " + 3·0 " $\frac{M}{100}$ " "	–	–	–	–	✦	✦	✦	✦	✦
" " + 2 " " + 2·0 " " "	–	–	–	✦	✦	✦	✦	✦	✦
" " + 3 " " + 1·0 " " "	–	–	✦	✦	✦	✦	✦	✦	✦
" " + 3·1 " " + 0·9 " " "	–	–	✦	✦	✦	✦	✦	✦	✦
" " + 3·2 " " + 0·8 " " "	–	–	✦	✦	✦	✦	✦	✦	✦
" " + 3·3 " " + 0·7 " " "	–	✦	✦	✦	✦	✦	✦	✦	✦
" " + 3·4 " " + 0·6 " " "	–	–	✦	✦	✦	✦	✦	✦	✦
" " + 3·5 " " + 0·5 " " "	–	–	–	✦	✦	✦	✦	✦	✦
" " + 3·6 " " + 0·4 " " "	–	–	–	–	✦	✦	✦	✦	✦
" " + 3·7 " " + 0·3 " " "	–	–	–	–	–	✦	✦	✦	✦
" " + 2 " " + 2 " $\frac{M}{1000}$ " "	–	–	–	–	–	–	✦	✦	✦
" " + 3 " " + 1 " " "	–	–	–	–	–	–	–	–	✦
" " + 3·1 " " + 0·9 " " "	–	–	–	–	–	–	–	–	Partially laked
" " + 3·2 " " + 0·8 " " "	–	–	–	–	–	–	–	–	Trace of laking
" " + 3·3 " " + 0·7 " " "	–	–	–	–	–	–	–	–	Trace of laking
" " + 3·4 " " + 0·6 " " "	–	–	–	–	–	–	–	–	–
" " + 4 " " (control)	–	–	–	–	–	–	–	–	–

✦ = Complete laking.

Times calculated from time at which tubes were placed in thermostat.

Of late years, it has been repeatedly demonstrated that the serum of an animal is able to exert a well-marked protective action against haemolytic agents on behalf of its own red blood corpuscles. We therefore tried the effect of substituting 1 c.c. of fresh sheep's serum in place of 1 c.c. normal saline in a series of tubes in which sodium cholalate in various strengths was used as a haemolytic agent; here it was found that the serum exercised a very marked inhibitory action on the haemolytic power of this substance. The results of such an experiment are seen in Chart 4.

It was found, for instance, that 1 c.c. of 0·1 M sodium cholalate, which when used alone was able to completely haemolyse the 1 c.c. of sheep's red corpuscles within five minutes, was, when used in conjunction with 1 c.c. of sheep's serum, absolutely devoid of haemolytic action. Such a result indicates very marked power indeed in the inhibition and prevention of haemolysis by the protective action of normal serum.

In examining the chart, one apparent contradiction will be noticed; in the case of the tube containing 3 c.c. 0·1 M solution of sodium cholalate, haemolysis was complete in twenty-two minutes. Such an amount, however, when used alone, is capable of completing haemolysis only after eighty minutes, so that in this particular case the serum has increased instead of diminished the haemolytic action.

This result is easily explained in the light of our former observations; if we assume that the amount of sheep's serum used was insufficient to neutralise the effect of the total amount of cholalic acid salt present, but had neutralised its equivalent amount, it is obvious that in this case a reduced amount of sodium cholalate would now be present in an active form, and since a weak dose (within certain limits) is more powerful than a stronger one, an increased action might naturally be expected.

Some sheep's serum was then inactivated by heating it at 56° C. for one hour; it was then tested to make sure that the process was complete; this was done by means of several controls against inactivated pig's serum which normally lakes sheep's red corpuscles. In a series of tubes similar to those used in the preceding experiment, 1 c.c. of the inactivated serum was substituted for the fresh material; it was found that the protective action was in no way diminished as the result of inactivation; indeed, if anything, it seemed more marked. It may, therefore, be assumed that in these cases the presence or absence of complement is immaterial in so far as the protective action of the serum is concerned.

A similar series of experiments were carried out using the sodium salt of choleic acid, and similar results obtained. Chart 4 shows these results epitomized.

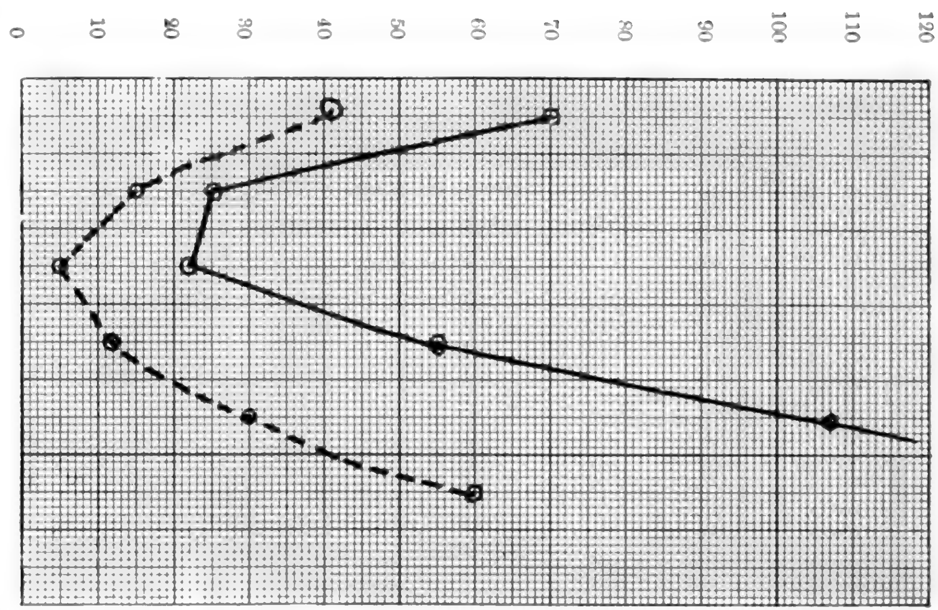
Chart No. IV

Description of Tubes	Results
1 c.c. sheep's r. b. c. + 1 c.c. sheep's serum + ... + 3 c.c. $\frac{M}{10}$ sodium cholalate...	**Complete haemolysis in 22 minutes**
„ „ + „ „ + 2 c.c. saline + 1 c.c. „ „	**Complete haemolysis after 5 hours**
„ „ + „ „ + 2·5 „ „ + 0·5 „ „ „	**Very slight haemolysis after 18 hours**
„ „ + „ „ + 2·7 „ „ + 0·3 „ „ „	No haemolysis
„ „ + „ „ + 1 „ „ + 2·0 $\frac{M}{100}$ „ „	No haemolysis
„ „ + „ „ + 2 „ „ + 1·0 „ „ „	No haemolysis
1 c.c. sheep's r. b. c. + 1 c.c. inact. sheep's serum + ... + 3 c.c. $\frac{M}{10}$ sodium cholalate	**Complete haemolysis in 25 minutes**
„ „ + „ „ „ + 2 c.c. saline + 1 „ „ „	**Almost complete haemolysis after 18 hrs.**
„ „ + „ „ „ + 2·5 „ „ + 0·5 „ „ „	**Mere trace of haemolysis after 18 hours**
„ „ + „ „ „ + 2·7 „ „ + 0·3 „ „ „	No haemolysis
„ „ + „ „ „ + 1·0 „ „ + 2·0 „ $\frac{M}{100}$ „ „	No haemolysis
„ „ + „ „ „ + 2·0 „ „ + 1·0 „ „ „	No haemolysis
1 c.c. sheep's r. b. c. + 1 c.c. sheep's serum + 1 c.c. saline + 2 c.c. $\frac{M}{100}$ sodium choleate	**Complete haemolysis in 113 minutes**
„ „ + „ „ + 2 „ „ + 1 „ „ „	No haemolysis
„ „ + „ „ + 2·5 „ „ + 0·5 „ „ „	No haemolysis
1 c.c. sheep's r. b. c. + 1 c.c. inact. sheep's serum + 1 c.c. saline + 2 c.c. $\frac{M}{100}$ sodium choleate ...	**Complete haemolysis in 140 minutes**
„ „ + „ „ „ + 2 „ „ + 1 „ „ „	No haemolysis
„ „ + „ „ „ + 2·5 „ „ + 0·5 „ „ „	No haemolysis
Controls	No haemolysis

Note.—The observations on these tubes extended over 24 hours.

After the sheep's r. b. c. were added the tubes were placed in a thermostat at a temperature of 37° C.

Fig 4. Chart No. 5.—Cholesterin Inhibition



1 c.c. sheep's r.b.c. + 3·6 c.c. Saline + 0·4 c.c. $\frac{M}{10}$ Sod. Cholalate

„ „ + 2·0 „ „ + 2·0 „ $\frac{M}{100}$ „ „

„ „ + 3·0 „ „ + 1·0 „ „ „ „

„ „ + 3·25 „ „ + 0·75 „ „ „ „

„ „ + 3·5 „ „ + 0·5 „ „ „ „

„ „ + 3·6 „ „ + 0·4 „ „ „ „

The dotted line indicates the time taken by the above tubes to lake.

The continuous line shows the haemolytic curve when 2 c.c. $\frac{M}{500}$ Cholesterin emulsion was added to each tube. The volume of each tube being constant at 5 c.c. for both experiments.

According to Liebermann, this inhibitory power of the blood serum is dependent on the serum-albumin. Iscovesco,[1] on the other hand, attributes it to the cholesterin normally present in blood serum. Gerard and Lemoine[2] have studied the anti-toxic action of cholesterin as applied to tubercular poisons. Ranson[3] has also demonstrated the anti-haemolytic power of cholesterin on certain chemical substances.

In view of these results, we have tried the effect of cholesterin on the haemolytic action of sodium cholalate. Using an 0·002 M emulsion of cholesterin, it was found that even in strengths of 0·0008 M solution it exercised a considerable anti-haemolytic action, and in the presence of weak doses of sodium cholalate was sufficient to entirely prevent a laking effect.

These results are demonstrated in Fig. 4, Chart 5. When using 0·4 c.c. 0·1 M sodium cholalate, the amount of cholesterin used (2 c.c. 0·002 M) was sufficient to delay the usual laking effect for about thirty minutes; with 0·5 c.c. 0·01 M there was a delay of seventy minutes, and in the case of 0·4 c.c. 0·01 M haemolytic action was totally abolished.

In all these experiments, the cholesterin emulsion and the sodium cholalate were incubated together at 37° C. for one hour previous to the addition of the red blood corpuscles.

The above are some of the results obtained with the substances mentioned; it is hoped to extend them at a later period. No theoretical considerations with regard to them have been advanced, since much more work must be done before any satisfactory explanation can be forthcoming; they are merely given as facts obtained as the result of repeated experiments.

Conclusions

Such bile derivatives as the sodium salts of cholalic, choleic, and glycocholic acids are capable of producing haemolysis in the ordinary way when strong doses are used, but exhibit marked peculiarities when present in considerably weaker amounts:—

1. It was found that under similar conditions the same haemolytic effect can be produced in a given time by widely divergent amounts of the salts. Between these two points lies what may be termed a more or less

1. 'Les Lipoides,' 1908.
2. Congrès de Médecine, 1907. *Soc. Médic. des Hospitaux*, Paris, 1907.
3. *Deutsche med. Wochenschr.*, 1901.

neutral zone, in which haemolysis is very considerably delayed depending on the relative amounts of haemolytic agent employed. The minimum dose giving the maximum effect in the shortest time we have designated the 'minimal-optimum haemolytic dose.' For instance, it was found that this minimal-optimum dose in the case of sodium cholalate was 0·1 c.c. 0·1 M solution; this quantity, mixed with 1 c.c. of 5 per cent. sheep's red corpuscles (the total volume being made up to 5 c.c.), gave complete haemolysis within five minutes. *Stronger* solutions gave a much *less* marked effect, a solution eight times as strong requiring one hundred and fifty-five minutes for a similar result, while a dose eighty times as large as the minimal-optimum dose required thirteen minutes to complete haemolysis.

2. In all these cases the addition of cholesterin produced a marked anti-haemolytic effect.

3. In a similar manner, the addition of fresh sheep's serum exercises a markedly anti-haemolytic action; in some cases, however, an apparent augmentation is in evidence. This is obviously due to the fact that the amount of serum used was unable to completely neutralise the laking action of the bile salts; this would result in a smaller relative amount of active haemolytic agent being present, and hence a more powerful effect would be seen (within certain limits), in accordance with the above observations.

4. This inhibitory action of serum does not depend on the presence of complement; an inactivated serum acts equally well.

We wish to thank Professor Benjamin Moore for advice and assistance in the above investigation.

THE PHARMACOLOGY OF APOCYNUM CANNABINUM*

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I. INTRODUCTION.

II. ACTION ON 1. Vascular System,
- (*a*) Heart.
  - (i) Frog.
  - (ii) Mammal.
  - (iii) Relative Toxicity of Apocynum.
- (*b*) Blood Vessels.
- (*c*) Blood Pressure.

2. Toxic effects.

3. Muscular System.

III. COMPARISON OF APOCYNUM CANNABINUM WITH OTHER MEMBERS OF THE CARDIAC GROUP OF DRUGS.

I

Apocynum is a member of the group of cardiac tonics, and is official in the United States. It has been credited with being the most powerful indirect diuretic known. Descriptions of the drug are given by Paine[1] and two Russians, Gliski[2] and Gvozdinsky.[3] In this country records[4] of patients suffering from pleuritic effusion have been given, under the influence of Apocynum it was thought that the fluid subsided 'rather quickly.' Hauseman[5] first suggested that Apocynum might contain a cardiac poison allied to the digitalin group, and in 1883 Schmeideberg isolated two so-called active principles, apocynin and apocynëin. Rose Bradford showed that its principal action was upon the heart. Sokoloff[6] showed that the drug caused slowing of the heart's action, 'enlargement of the pulse' wave and marked rise of blood pressure. Petteruti and Somma[7] found different results were obtained according as to whether they used the decoction or the tincture, the decoction acting chiefly on the stomach and intestines causing catharsis and emesis, when this action was

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absent there was diuresis and acceleration of the heart beat. The tincture was stated to be unirritating to the gastro-intestinal tract even in large doses. The emetic and cathartic action of the decoction was attributed to the admixture of the bitter fibre of the wood with the bark of the root.[8] Further experimental work was carried out by Dortschewski,[9] Klopotovitch[10] and Lapshin. Since my experiments have been completed, I find that Laidlaw and Dale have been working with a crystalline active principle isolated by H. Finnemore.[11] Some apology is, therefore, necessary for the late publication of this paper.

The preparations used in my experiments were (*a*) Tinctura Apocyni Cannabini. One part of the root to ten parts of 60 per cent. alcohol. (*b*) Apocynin.

The animals employed were frogs, rabbits, dogs and cats. Frogs were used for simple injections into the dorsal lymph sac, and for demonstrating the action of the drug on striated and unstriated muscle, the gastrocnemius muscles and a sectional ring of the stomach being used respectively. Perfusion experiments were also performed by tying a cannula into the hepatic vein. The effect of the drug on the heart in situ, and also in connection with recording apparatus, was also observed. The frogs were always pithed, except those used for simple injections.

Observations were made with rabbits on the effects of injecting both large and small quantities of the drug; the action on the vascular system was also determined.

Dogs and cats were employed for obtaining cardiometer and oncometer records; experiments on the urinary flow were performed on dogs.

Chloroform, A.C.E. mixture and urethane were used as anaesthetics. In a few cases no anaesthetic was used, the animals being pithed.

II. Action

1. *Vascular System.*

(*a*) Heart. (i) Frog. The most important action of Apocynum is seen in its effects on this organ. In a general way Apocynum acts on cardiac muscle in the same manner as it acts on both striated and unstriated muscle. The tonus is always increased, the systole and diastole are greater in amplitude. Cardiac slowing is a marked feature.

On placing on the heart a few drops of a crude extract of the root of Apocynum a marked effect is immediately apparent. The beat is slowed and a clear rise of tonus occurs.

Such experiments are of little value, since the effect of a drug applied to the outside of the heart is not necessarily the same as when the drug is given by the circulation, but they suffice to indicate the probable effect.

Fig. 1 shows the movements of a frog's heart recorded by the suspension method,[5] so that the systole is represented by the upstroke. A saturated alcoholic solution of Apocynin was used for injection into the subcutaneous tissues. Section I shows the normal beat. In II is seen an increase of systole and diastole with some slight slowing after a subcutaneous injection of 10 minims of the Apocynin solution. An increased effect is noticed in III and IV after applying the solution directly to the heart. Section V gives the results of placing crystals of Apocynin on the heart. The greatest amplitude and most marked slowing of the beat is seen in the first part of VI, after this, the beat gradually quickens until it becomes very quick and irregular (delirium cordis) and the heart dies in systole.

The experiment illustrated in fig. 1 is particularly interesting, as it shows that the substance Apocynin is active, and that it causes an effect like the tincture. It is also of some interest to observe the delirium cordis, as this is a condition particularly difficult to obtain in the frog.

After a subcutaneous injection of $1\frac{1}{2}$ minims of tincture of Apocynum in the frog, a similar series of events occurred, and death ultimately occurred in systole.

These simple experiments show that the crude extract of Apocynum, the glucoside Apocynin, and the ordinary standard tincture, all give rise to the same series of effects in the frog. The heart beat is slowed and increased in amplitude, later in the action the beat becomes quicker and irregular and death occurs in systole. The development of these effects was also seen in tracings of perfused frog's hearts.

(ii) Mammalian heart. Experiments were undertaken to demonstrate the effect of small doses of Apocynum on the isolated mammalian heart.

Fig. 2 represents the records of an experiment in which the drug was introduced (5 c.c. Tinct. Apocyni, 1 in 6,000) into the side tube of the perfusion apparatus so that after considerable dilution with the Ringer's solution in the apparatus all the drug would rapidly pass through the isolated heart. In other words, five minutes after the injection, no Apocynum would be in the circulating fluid. Section I shows the heart beating normally. II shows the effect five minutes after injection; the rate of the beat is the same, but the amplitude of the systole is increasing. Tracings III and IV show still later effects, 20 and 25 minutes after injection the rate of beat still remains about the same, 42 to the minute. In V, a record taken 30 minutes after injection, the heart is beating much more slowly and ventricular beats are being lost, so that two auricular beats are shown to each ventricular beat, and in the later portion of the tracing the ventricular beats entirely cease for a time. This stage is rapidly succeeded by that of delirium cordis in which the heart beats very rapidly and the tonus of the cardiac muscle rises (VI, VII, and VIII). The heart ultimately stops in systole (IX and X) fifty minutes after the injection.

It may be noted here that the death of this heart, which is typical of many experiments, results from a very small dose of the drug, in spite of

I. II. III. IV. V. VI.

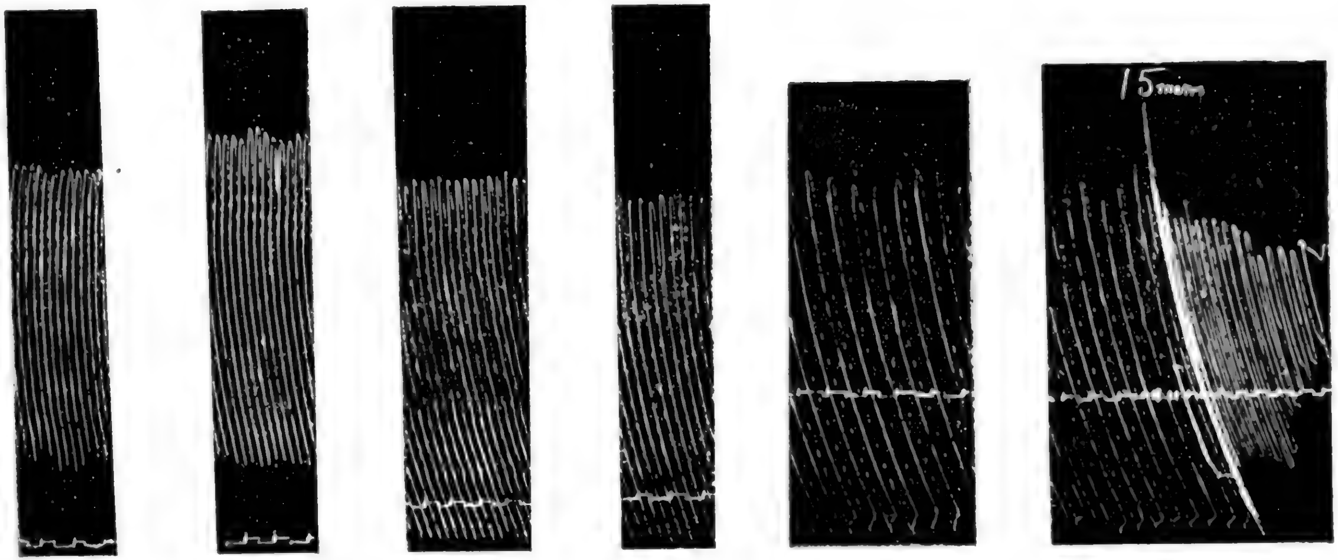


FIG. 1.

Heart. Frog. I.—Normal beat.

II.—After subcutaneous injection of ♏ X alcoholic solution Apocynin.

III.—After applying solution of Apocynin directly to the heart (1st time).

IV.— " " " " (2nd time).

V.—After applying crystals of Apocynin directly to the heart.

VI.—Continuation of V. Delirium cordis and death.

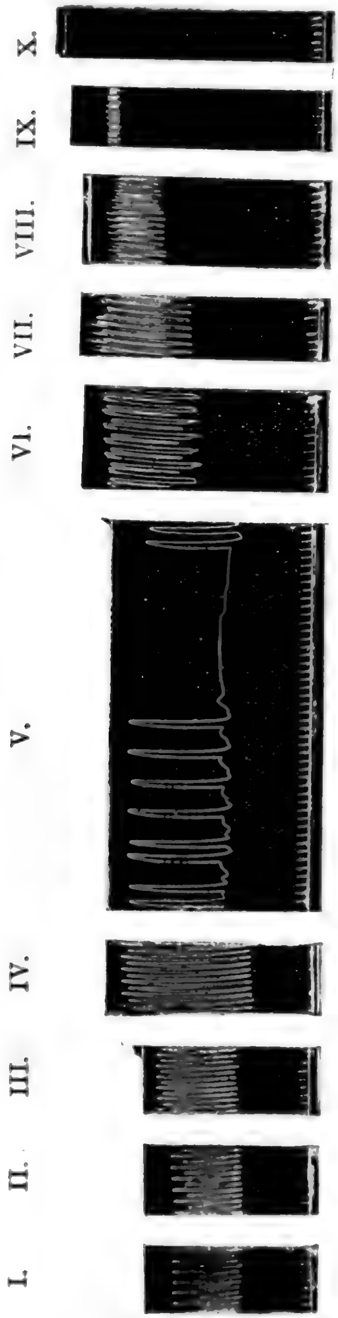


Fig. 2.

Rabbit's Heart. Isolated. I.—Normal beat, perfused with Ringer's solution.

II.—Five minutes after injecting by side tube 5 c.c. 1 in 6000 Tincture Apocynum.

III and IV.—Later stages.

V.—Dropping of ventricular beats.

VI and VII.—Rising tonus.

VIII, IX and X.—Arythmia, delirium cordis, and death in systole.

Scale $\frac{2}{3}$ linear.

the facts that the administration was of short duration, that the small quantity of fluid containing the drug was quickly washed away by the constant flow of fresh saline solution during the whole of the experiment, and that only one dose was given. Evidently not all the drug administered is removed by the constantly flowing saline solution, but it would seem that some part of the dose remains behind in a kind of fixed combination with the cardiac muscle, and that it is this portion of the drug which is responsible for the production of the ultimate toxic effects. This may be taken as an example of cumulative action. I would like to compare this drug with strychnine or atropine which, when administered, induce a certain definite action but are rapidly excreted. Apocynum, on the contrary, is fixed by the heart, and continues to produce an effect on the heart when no drug is present in the coronary circulation.

In other experiments the heart was perfused with the Ringer's solution, but after a short time this was replaced by a saline solution of the drug, so that the heart was bathed during the remainder of the experiment with a diluted tincture of Apocynum (1 in 6,000): this is a condition which would approximate in a remote degree to that prevailing during life while any drug was continuously and regularly administered, and which was absorbed gradually into the circulation. Such experiments showed what may be regarded as the therapeutical action of the drug, systole was augmented, diastole prolonged, and the tonus was unaltered; later the poisoning action was shown by the acceleration present. The acceleration was further increased; so rapid was the heart that diastole was incomplete, the heart beats followed one another too rapidly for complete diastole to occur. Marked arhythmia was present and tonus was increased, a condition which only occurs when the heart is beginning to die. In some tracings, groups of two following beats were seen, so that there is a greater interval between each group of two following beats than between the individual beats of each group. This is a condition which appears in that form of irregular pulse known by the names of coupled beats, allorrhythmia, pulsus bigeminus (twin pulse), and pulsus biferiens. It is well known clinically that this type of pulse may at any time appear during the administration of Digitalis, especially when the effect is becoming cumulative, and it seems also that this is one of the toxic effects of Apocynum. Experimentally, this form of heart beat is only seen when the heart muscle has been rendered 'hyperirritable' by a drug of the digitalis group. One heart died in a state of extreme contraction, having been at work for three hours.

Some further experiments were performed with the cardiometer. Cats anaesthetised with urethane were used for this purpose, and the drug was given directly through the circulation. Apocynum in small doses increases the output of blood from the heart; the effect comes on slowly, but is very prolonged. This effect is, of course, the true criterion of a cardiac tonic, an increased cardiac output being the true sign of stimulation.

The action on the rate of the heart beat varies according to the stage of action of the drug. In the therapeutic stage the heart is slowed, and is quickened in delirium cordis, which constitutes the poisoning stage of the drug.

The slowing effect of Apocynum on the heart, as indicated by the pulse and also the influence on the respiration, was shown generally by the following simple experiment, for which I am indebted to Professor Dixon. A small-sized dog was given 5 minims of Tincture of Apocynum subcutaneously. The data given in the following table indicate the changes in the pulse rate and respiration :—

Time	Pulse	Respiration
4.30	114	20
4.45	120	16
4.55	76	14
5.0	96	16
5.6	108	20
5.15	108	16

Similar results were obtained on man.

(iii) Relative toxicity of Apocynum on the heart. For this purpose solutions of various strengths of the substance Apocynin and of the Tincture of Apocynum were injected into the dorsal lymph sac of the frog; Dixon and Haynes[12] having previously shown that this forms a suitable means for determining the relative activity of the cardiac tonics.

Effects of injections of Apocynin:—

Exp. I. 5.30 p.m. 1 c.c. of an 0·5 solution of Apocynin was injected into the dorsal lymph sac of a frog weighing 19 grammes.

5.35 p.m. Reflexes less brisk, respiration quickened, animal crawls about but does not sit up.

5.55 p.m. Respiration jerky, irregular groups of two or three respirations occur in quick succession. Responds only to forcible pricking stimulation.

6.20 p.m. By this time the frog had completely recovered.

Exp. II. 3.45 p.m. 2 c.c. of an 0·5 solution was injected into the dorsal lymph sac of a frog weighing 12 grammes.

4.45 p.m. By this time the frog had fully recovered, having been only sluggish for the first half hour after injection.

Exp. III. 3.50 p.m. A frog weighing 14 grammes was injected with 3 c.c. of 0·5 solution.

3.56 p.m. Frog motionless in dorsal position, pupils widely dilated, responds to pinching toe.

4.10 p.m. Breathing very feeble, struggles at times.

4.25 p.m. Turned on to its feet, pupils contracted.

5.0 p.m. Recovered but slightly, slow in its movements.

Exp. IV. 5.0 p.m. A frog weighing 16·5 grammes was injected with 3 c.c. of a 1·25 per cent. solution of Apocynin.

5.5 p.m. Heart beats 60 per minute.

5.13 p.m. Heart beats 48 per minute.

5.15 p.m. Animal very feeble, does not respond to stimulation. The heart was now exposed, the ventricle was feebly contracting and ceased in diastole at 5.25 p.m. The auricles and sinus venosus still contracted in groups of three beats at a time.

5.35 p.m. Dead.

Effects of injections of the Tincture of Apocynum:—

Exp. I. 4.45 p.m. Frog weighed 20 grammes. 15 minims of the tincture injected.

4.50 p.m. Animal helpless in dorsal position.

5.5 p.m. Slight corneal reflex.

5.20 p.m. Dead.

Exp. II. 4.40 p.m. Frog weighed 12·5 grammes. 7 minims of the tincture injected.

5.5 p.m. Crawls with difficulty.

5.15 p.m. Slight corneal reflex.

5.20 p.m. Dead.

Exp. III. 4.38 p.m. Frog weighed 15 grammes. 3 minims injected.

5.5 p.m. Recovers from the dorsal position with difficulty.

5.30 p.m. Dead; ventricle firmly contracted.

Exp. IV. 3.53 p.m. Frog weighed 17 grammes. 2 minims injected.

4.30 p.m. Dead.

Exp. V. 3.55 p.m. Frog weighed 12 grammes. 1 minim injected.

5.25 p.m. Leaps and falls on its back, recovery difficult, tumbles over.

5.30 p.m. Crawls with difficulty.

5.35 p.m. No recovery from dorsal posture, moves limbs. Hind limbs paralysed before upper ones.

5.45 p.m. Dead.

These simple injection experiments serve to demonstrate the comparative inactivity of Apocynin, and the toxicity of the tincture of Apocynum upon the heart. Smaller amounts than one minim of the tincture had no fatal effect. One minim may therefore be regarded as the minimum lethal dose for the standard tincture of Apocynum.

From the results of two of the experiments in each of which the frogs died in 35 minutes, one from the effects of Apocynin and the other from the tincture of Apocynum, it appears that 3 c.c. of a 1·25 per cent. solution of Apocynin is about equivalent to 15 minims of the tincture of Apocynum in toxicity. Approximately the 1·25 per cent. solution of Apocynin is

$3\frac{1}{2}$ times less toxic than the tincture of Apocynum. For the sake of comparison, the following table of the minimum lethal doses of the most commonly used cardiac tonics is appended:—

Drug	M.L.D. (in minims) per 100 grammes of frog
Tinct. Digitalis (13)	12·5
Tinct. Strophanth. (13)	1·47
Tinct. Scillae (13)	12·5
Tinct. Apocyni	8·33
Apocynin (1·25 per cent. sol.)	29·15

From this it seems that the tincture of Apocynum is about one and a half times more toxic than the tinctures of Digitalis and Squill, and nearly six times less toxic than the tincture of Strophanthus. The substance Apocynin possesses a fair degree of activity, although it is the least toxic of the series in the above table.

All the experiments show that Apocynum acts on the heart somewhat similarly to Digitalis in causing slowing of the heart beat. The principal action is to raise the tonus or condition of continued contraction of the muscle of the heart, especially in toxic doses the ventricle may be so firmly contracted that it appears quite pale. This is well seen in the frog's heart and also in the mammalian heart when mounted on the perfusion apparatus. If a sufficiently small dose of the drug is administered the first effect usually noticed is a gradual increase in the extent of the heart beat, the increase in the excursions of the recording lever increasing until the vertical height of the tracing becomes two or three times greater than it was before the drug was administered. This increase in amplitude of beat is accompanied by a decreased rate of beat, which is more marked in Batrachian than in Mammalian hearts. The amount of slowing produced varies from rather less than half the original rate up to not more than three times as slow. The heart at this stage is quite regular, relaxation is more complete and so is contraction; under favourable circumstances the contraction of the auricles may equal the contraction of the ventricles in extent. This condition of affairs should represent the most perfect therapeutical effect of the drug.

When this is being most completely manifested a change in the character of the beat more or less suddenly takes place, some quickening and irregularity occur. The ventricular portion of the beat may be dropped, and each chamber of the heart in mammals may contract or not independently of the rest without any order or sequence. This condition of irregularity, combined with extreme rapidity of beat, constitutes the

condition of delirium cordis, and is a toxic effect of the drug. In mammalian hearts the delirium ends in fibrillary twitching. Most of the slowing of the heart beat is due to the action of the drug on the nerve terminals of the vagus in the heart muscle; for if these terminals be paralysed by atropine the slowing produced by the Apocynum is much less decided, although there is a very slight amount seen, which must be due to the direct action of the drug on the muscular tissue of the heart. The slowing effect gives place to a stage of quickening; during this quickening the vagus nerve appears to be paralysed, since no slowing of the beat can be obtained either by electrical stimulation of the nerves or the injection of a drug such as pilocarpin or muscarin, which are known to excite vagal endings. Now, Apocynum is not a paralytic drug; so far as we know, it depresses no nerve endings, and its effect in preventing vagus inhibition has a more ready explanation than the supposition that it paralyses vagal endings like atropin. Apocynum acts essentially on heart muscle by increasing its irritability, and in the later stages of its action, this irritability shows itself by marked acceleration. The vagus nerve is intact, but such is the irritability of the cardiac muscle that it has no longer the power to hold it in check. The acceleration of beat, accompanied in its later stages by irregularity, stimulates that part of the heart known as the excito-motor area, and so the rapidity of beat is increased continually until fibrillary twitchings occur. The amount of work done by a heart under therapeutic doses of Apocynum is increased, or in other words, the output of blood is increased. The increase in the amount of work is due to the combined action of the Apocynum on the nervous and muscular structures of the heart. The increased force of the beat is due to the stimulant effect of the drug on the muscle alone, the stimulant effect on the vagus endings produces some inhibition, so that the heart becomes slower, the diastole is prolonged, and the heart is more completely filled with blood. The systole is also increased, not only in strength, but also in length, so that there results a more complete emptying of the heart in systole.

(*b*) Blood Vessels. The general effect of Apocynum on the blood vessels was shown by the perfusion of a pithed frog. The hepatic vein of a fair-sized frog was displayed, and a small cannula tied into it. This cannula was then connected with a simple perfusion apparatus. One leg of the frog was cut off below the knee, so that the perfusing fluid as it circulated through the body of the frog dripped from the cut end of the limb. The drops were counted at short intervals for periods of one

minute; the frog was first perfused with normal saline solution, and then with a solution of 1 in 60 Tinct. Apocyni. This experiment distinctly showed that Apocynum is a powerful vaso-constrictor, as it diminished the flow from 31 to 6 drops per minute. This effect is a peripheral one, as the brain and spinal cord of the frog were destroyed, so that no central vaso-motor influence was at work. It may be noted that this diminution of flow was not due to any decrease of the action of the heart, as at the end of the experiment this organ was still beating vigorously. The vaso-constriction must be due to the direct action of the drug on the nerve endings or muscular coats of the blood vessels.

The vaso-motor effects of Apocynum were further demonstrated by another experiment.

The record in Fig. 3 was taken from a pithed cat and the blood pressure recorded in the usual way with a mercury manometer, the changes in the intestinal vessels were recorded by an oncometer. Two c.c. of a 1 in 4 solution of the tincture were injected by the side tube into the jugular vein, and at once a very marked rise of blood pressure is seen. This was accompanied by a lowering of the oncometric curve, which indicated a diminution of the intestinal volume owing to the powerful contraction of the intestinal vessels, after this the volume increased owing to the dilation of the organ as a whole, no doubt brought about by the great increase in the cardiac output. The volume of the intestine at about the maximum blood-pressure is the resultant of two factors, namely increase of size from the distension caused by the greater blood-pressure, and decrease of size owing to the great vaso-constriction as shown by the volume pulse. During the supervention of delirium cordis the intestinal volume again underwent decrease; owing to the marked fall in blood-pressure the intestine was less distended and the vaso-constriction factor became evident. At the end of the experiment the blood-pressure fell rapidly and suddenly to zero, and intestinal pallor was noticeable. The administration of Atropin had no effect on the final result as the vagal influence had already been annulled by the Apocynum: this is due to the increased irritability of the heart muscle which can no longer be controlled by the vagus.

In pithed dogs similar experiments were performed with like results. Further experiments were undertaken on the renal vessels, and the same type of constriction as in the splanchnic vessels occurred. In every case, then, in which this drug was perfused through the vessels or given internally, vaso-constriction resulted. To determine whether the drug acts on muscle or nerve endings, the pulmonary vessels of a mammal were perfused. The first effect was a short dilatation, followed by a profound and prolonged constriction; adrenalin, which was first injected, having no influence on the blood flow. Therefore, since we know that the pulmonary vessels are not innervated, the action of Apocynum must be on the muscle fibre. The constriction of the blood vessels produced by Apocynum is well marked, not only during its therapeutic action, but also during the development of its toxic effects. Finally, all the experiments,

such as perfusing a frog's body or a mammal's lung, or recording changes in the volume of kidneys, loops of bowel, and forelimbs, show very conclusively that this drug is a powerful vaso-constrictor. And it is to be noticed that this effect is a peripheral one, due to the direct action of the drug on the muscular tissues of the blood vessels, since the constriction still takes place when the vaso-motor centre in the medulla is destroyed and also when the drug is perfused through blood vessels which have no

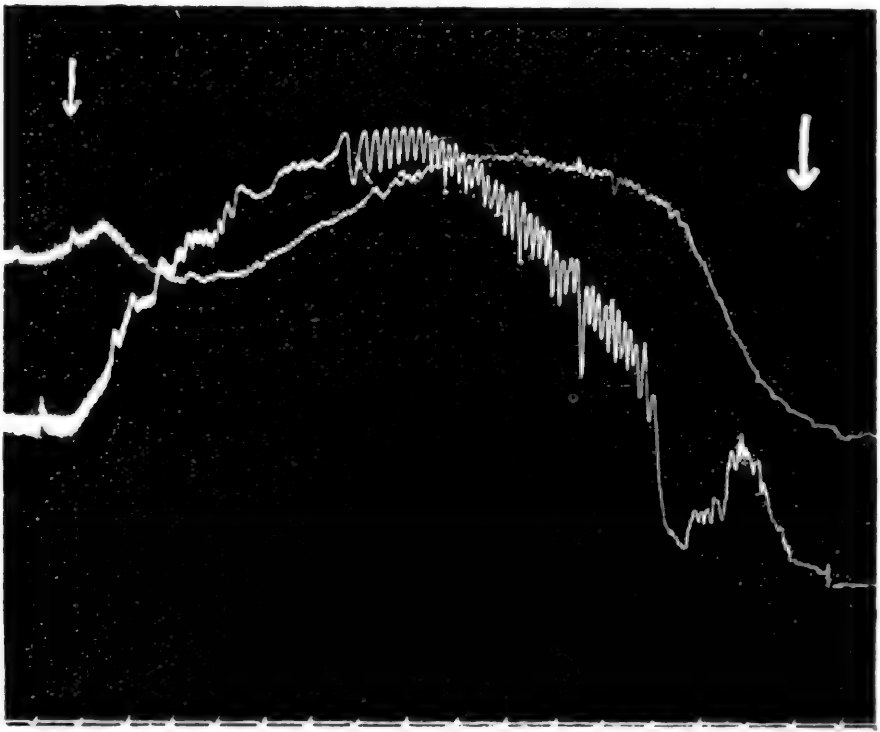


FIG. 3.

Cat—pithed. Artificial respiration. Upper tracing taken from intestine enclosed in oncometer; lower tracing records blood pressure. Delirium cordis—death.

2 c.c. ½ strength Tinct. Apoc. introduced per jugular vein at 1st arrow. Atropin at 2nd arrow.

Time 20 seconds.
Scale ⅔ linear.

vaso-motor nerve endings; the integrity, or otherwise, of the nervous system makes no difference to this action of Apocynum on the vascular tissues.

(c) Blood Pressure. In every experiment where the blood pressure has been taken, certain changes in the tracings of the blood pressure have always been recorded. In a pithed dog an immediate rise of blood pressure

is recorded after the injection of 1·5 c.c. of Tinct. Apocynum. The slowing of the heart beat was reflected in the tracing. This is the therapeutical effect of the drug, but in this case it quickly gave place to rapidity and irregularity of the heart beat and a further rise of blood pressure; this condition is entirely due to the enormous dose of the drug given; it is, of course, never seen when the dose is moderate. The delirium cordis is increased by the further injection of 5 c.c. of the tincture, and the blood pressure ultimately falls to zero.

This experiment was typical, and the same results with similar effects are obtained in cats and rabbits after massive doses of the drug.

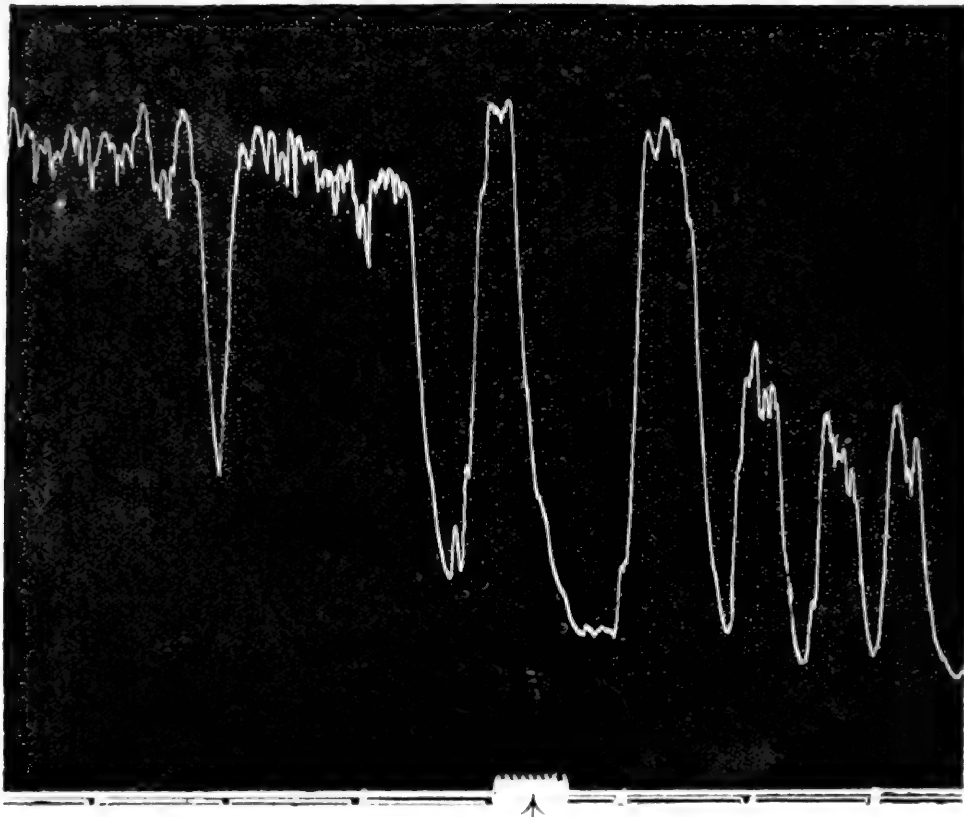
The following experiment shows the effect of the drug on the blood pressure with the heart atropinised. A rabbit was pithed and connected with the artificial respiration pump; the blood pressure was recorded in the usual way. After two injections of 1 c.c. of an 0·5 per cent. solution of Atropin, the cardiac terminations of the vagus were paralysed, and, consequently, the heart rate increased and the blood pressure rose to some extent; some slowing of the heart beat and a rise of blood pressure occurred after a hypodermic injection of 20 minims of Tinct. Apocynum: delirium cordis eventually prevailed. In this experiment the slowing produced was entirely due to the influence of the drug on the heart muscle alone.

The action of Apocynum on the blood pressure is due to the combined effect of two or more factors. In all the experiments the general effect of Apocynum has been to produce a rise of blood pressure, and this occurs in spite of the slowing of the heart due to the stimulation of the vagal endings. The rise in blood pressure depends on two factors, (1) the increased output from the heart per minute, (2) vaso-constriction. It is when these factors, namely, the cardiac slowing, with the increased strength of beat resulting in augmented output, and vaso-constriction are most in evidence that the drug produces its therapeutical effect. The rise of blood pressure is thus the mean result of the above changes. Sometimes before the typical rise ot blood pressure occurs there is a fall in the normal blood pressure; this is due to the sudden stimulation of the vagus overshadowing for the time being the other effects of Apocynum. I have more than once in this way seen the heart die straightway from vagal inhibition. When delirium cordis supervenes the blood pressure undergoes the most extreme variations, or it may at once fall to zero

Fig. 4. A cat was anaesthetised with urethane and the blood-pressure recorded in the usual manner. The tracing shows a very perfect delirium cordis, in which the vagal influence is entirely eliminated as no slowing effect is produced by the injection of 5 c.c. of an 0·5 per cent. solution of Pilocarpin. Three c.c. of Tinct. Apocynum had previously been injected.

With large doses the irritability of cardiac muscle rapidly increases, and the heart beat quickens; and hence in this stage the blood pressure rises greatly, both on account of increased cardiac output and vaso-constriction. Still later, as the irregularity of auricles and ventricles further increases, they take on their own rhythm; sometimes the auricles

FIG. 4.



Pilocarpin 5 c.c. 0·5 per cent. solution.

Cat—Urethane. Shows perfect delirium cordis in which vagi have no control—after 3 c.c. of Tinct. Apocyni. No slowing effect after pilocarpin.

Full Scale.

may force blood into the ventricles during diastole of the latter, in which case when the ventricles contract the blood pressure rises. Sometimes there is no sequence between auricles and ventricles, and then ventricular contractions fail to keep up blood pressure. At this stage records of blood pressure show great variations, sometimes bounding up to 50 mm. of mercury for half a minute, and sometimes falling almost to zero.

Besides the action on the heart and blood vessels, Apocynum has some influence on the kidneys. The kind of action the drug has on the excretory function of the kidney is indicated by the following experiment:—Urethane and morphia were injected into a dog, anaesthesia being maintained in the usual way by the A.C.E. mixture. The blood pressure was recorded by a mercury manometer and the artificial respiration pump connected with the trachea. The abdominal cavity was opened, and one ureter was dissected out and a cannula inserted. The urine was collected in a measuring glass every two minutes Sixteen and eighteen drops of urine passed in equal periods of time, with a rise of blood pressure; twenty-three and twenty-one drops of urine passed in equal periods of time, after the injection of 1 c.c. of a 1 in 10 solution of Tinct. Apocynum.

Observation		Amount of Urine flowing in two minutes	
1	...	2·3 c.c.	
2	...	2·2 c.c	
3	...	2·1 c.c.	
4	...	1·7 c.c.	
5	...	1·8 c.c.	
6	...	1·3 c.c.	
7	...	1·0 c.c.	
8	...	1·2 c.c.	
9	...	0·8 c.c.	
			0·5 c.c. (1 in 2 sol.) Tinct. Apocynum injected
10	...	0·6 c.c.	
11	...	0·4 c.c.	
12	...	0·9 c.c.	
13	...	1·1 c.c.	
14	...	1·3 c.c.	
15	...	1·2 c.c.	
16	...	1·3 c.c.	
17	...	0·8 c.c.	
18	...	0·8 c.c.	urine blood-stained
			Sod. sulphate solution injected by side tube
19	...	1·2 c.c.	
20	...	3·2 c.c.	
21	...	3·2 c.c.	
			1 c.c. Tinct. Apocynum injected
22	...	4·3 c.c.	
23	...	5·5 c.c.	
24	...	1·5 c.c.	
			1 c.c. Tinct. Apocynum injected
25	...	1·2 c.c.	Delirium cordis
26	...	0·2 c.c.	
			Death
27	...	0·0 c.c.	

It is to be noticed that the increase of urinary flow is coincident with the rise of blood pressure. Apocynum is sufficiently irritant in character to cause the appearance of blood in the urine. The administration of a saturated solution of Sodium Sulphate by the jugular vein causes a very much greater diuresis than Tinct. Apocynum; the latter is very feeble as a direct diuretic.

From this it appears that Apocynum causes a feeble increase of urinary flow in the healthy animal; if the action is continued long enough and the dose is sufficiently large, haematuria may result. The slight diuresis is not due to any direct action on the kidney, but is due to the increase of blood pressure and passage of a greater quantity of blood through the kidney; because the flow of urine in normal animals always bears a direct relationship to the condition of the renal vessels and blood pressure. If the renal vessels are much contracted the flow of urine diminishes, but if they are only slightly contracted and the blood pressure is considerably raised, the flow of urine is slightly increased. In patients with cardiac disease and a failing heart, when there is back pressure and venous congestion and oedema of the kidneys as well as in other organs, little urine is secreted. The kidney is particularly sensitive to venous blood, and ceases to excrete as soon as the oxygen reaches a certain point. Now it is just in these cases that Apocynum produces so great a diuretic effect. It acts, then, not by any direct influence on the kidney, but by improving the condition of the circulation and so sending arterial blood once more to the kidneys. The kidney vessels are at the time of diuresis actually constricted, but this constriction must not be very complete, otherwise the diuresis will lessen. As in the case of Digitalis, the increase in urinary flow is due neither to the rise of blood pressure nor to any action on the excreting arrangements of the kidney, but to the increased quantity and quality of the blood which is driven through the kidney owing to the greater force of the heart beat. The diuretic effect of Apocynum is a purely dynamical one, and is quite different to the action of such a diuretic drug as Caffeine, which produces dilation of the kidney vessels as well as a rise of blood pressure; the very marked flow of urine which follows an injection of a solution of Sodium Sulphate (see table) is an example of a third method of diuretic action, and occurs probably because the salt solution has some kind of direct action on the renal epithelium.

2. *Toxic effects.*

The drug has a penetrating bitter taste, which gradually increases until a feeling of nausea supervenes. Large doses have a very toxic effect on the mucous membrane of the alimentary canal.

Experiment. A rabbit weighing 1,350 grammes was taken, and for eight consecutive days 5 minims of Tinct. Apocynum with 10 minims of normal saline solution were injected into the marginal vein of the left ear. On the sixth and seventh days the animal was noticeably ill and exhausted; it was constantly vomiting and had lost some weight. Death occurred suddenly on the eighth day.

On examination the most obvious changes had occurred in the alimentary canal. The whole of the stomach and intestines were closely marked with haemorrhagic spots and areas, chiefly situated on the side of the gut distal to the attachment of the mesentery. On opening the stomach and portions of the intestines these haemorrhagic areas were found to correspond with ulcers of various shapes and sizes in the mucous membrane. The ulceration was general throughout the whole length of the alimentary canal, from the lower end of the oesophagus to the rectum. The ulcers varied in size from 2 cm. square, with complete denudation of epithelium, to mere punctures. These small punctate ulcers easily perforated on washing portions of the gut through from a pressure tap, when the water sprayed out in many small streams.

The main blood vessels appeared to be normal, but some small white sclerotic patches were noticed in the aorta a short distance above the semi-lunar valves. The heart was larger than would be expected. Another small sclerotic patch was noticed in the wall of the left ventricle just below the semi-lunar valves. The remainder of the organs appeared healthy.

Experiment. A second rabbit was used weighing 1,200 grammes. Two minims of Tinct. Apocynum were injected into the ear veins at intervals of one, two or three days over a period of seventy days altogether. At the end of this time the animal was killed. The left ear was oedematous and swollen to three or four times its ordinary size. The left eye was very prominent, and the conjunctiva extremely swollen and oedematous.

On opening the thorax, some slight amount of fluid was found in the pleural sacs, both lungs were oedematous. The pericardial sac was distended with fluid. There was no increase of fluid in the abdominal cavity.

One other effect of Apocynum deserves mention here. The mucous membrane of the alimentary canal, like all other plain muscle in the body, is stimulated; peristalsis is increased, and may give rise to nausea, vomiting, and diarrhoea. This effect is, no doubt, partly local; stimulation of the mucous membrane causing local reflexes through Auerbach's plexus, with resulting peristalsis. But this is not the whole explanation, since Apocynum still causes some increased movements of the alimentary canal even after absorption, or the same effect can be induced by injecting the drug directly into the circulation of an animal.

The results of the experiments serve to illustrate the extremely irritant action of Apocynum on the tissues of the body. In the first rabbit to which the larger dose was given, the intestinal ulceration may be regarded as a secondary lesion occurring at the points of elimination of the drug into the intestine. As far as the naked eye appearances go, the frequent haemorrhagic areas point to profound changes in the blood vessels of the intestine, and these changes are certainly correlated with the appearance of the patches of sclerosis seen in the aorta.

Further evidence of the influence of Apocynum on the vascular system is afforded by the lesions found in the case of the second rabbit; these were localised oedema, pulmonary oedema and slight hydrothorax, and distinct hydropericardium.

3. *Muscular System.*

(*a*) Striated muscle. The action of Apocynum on muscle is comparable to that of Veratrine and Barium. The most characteristic action of Apocynum on striped muscle is the delayed relaxation and constantly increasing rise of tonus; in this respect Apocynum exactly resembles Digitalis and Squill. It can be shown that this drug has the same type of action on all forms of muscle, but that the effect on the more delicate cardiac muscle overshadows the others.

(*b*) Unstriated muscle. This is thrown into a state of prolonged contraction, and the tonus is also raised so that in isolated preparations the death of the muscle occurs in contraction. Plain muscle throughout the body is affected in this way. The typical effect on this tissue may be simply observed in ring preparations of the frog's stomach, in which the movements are recorded by the suspension method, so that the down stroke of the tracing indicates the contraction (systole) of the preparation.

Apocynum applied directly to the preparation causes a decided contraction, and the tonus of the muscle is permanently raised; and this

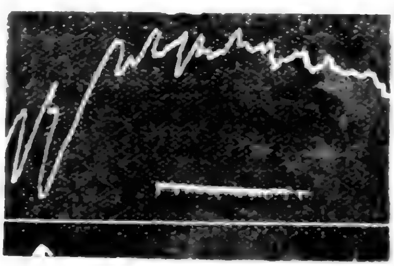
effect is obtained in spite of the fact that the tincture which was here employed contains alcohol, the action of which is to reduce tonus.

This effect on plain muscle may be regarded as representing the type of action occurring in all plain muscle throughout the body. I would observe here, however, that the alimentary canal shows irregular colicky contractions, the spleen and bronchioles are induced to contract, there is marked vaso-constriction and the tonus of the bladder and uterus is increased. The effect of Apocynum on the uterus was determined on the isolated uterus of a cat.

Experiment. Fig. 5. The uterus was suspended with its lower end fixed in an oxygenated saline solution and the movements recorded by suitable levers. The introduction into the saline solution of a trace of tincture of Apocynum causes a great increase in the muscular tonus. The peristaltic movements become less and less and ultimately cease, but the muscle remains in a state of tonic contraction. The uterus in this case was a pregnant one, and 0·2 c.c. of the tincture was administered at the arrow mark.

The type of action shown in the above experiment is very similar to that of digitalis, except that the effect of Apocynum on the uterine muscle is of a much more intense character.

FIG. 5.



Pregnant uterus of Cat. 0·2 c.c. Tincture Apocyni given at arrow produces rise of tonus and decrease in size of contractions. Death in systole.

Full Scale.

III. COMPARISON OF APOCYNUM CANNABINUM WITH OTHER MEMBERS OF THE CARDIAC GROUP OF DRUGS

Apocynum will be compared only with Digitalis, Strophanthus and Squill.

The experiments show that, of the four drugs, Apocynum stands second in relative toxicity on frogs' hearts. Strophanthus has the greatest toxic action. Digitalis and Squill, which are about equal in toxicity, have a less toxic action than Apocynum.

Apocynum has a greater tendency to cause delirium cordis than any of the other three drugs; in several experiments delirium cordis set in almost immediately after the administration of the drug, and in others no so-called therapeutical effects were obtained, also no tracings show generally such wide variations of blood pressure at this stage as those obtained from Apocynum.

This drug is the most irritant of the four towards mucous membranes, as shown by the intense ulceration produced in the alimentary canal after the intravenous injection of moderate doses of the drug. Strophanthus has been shown to be the least irritating to mucous membranes, and is in consequence the most readily absorbed. Apocynum is the most powerful vaso-constrictor of this group of drugs, and its property of constricting the blood vessels stands in direct relationship to what is really the specific action of the drug, that is its ability to increase the tonus of muscular tissue of every kind. In connection with this action, a greater rise of blood pressure is produced by Apocynum than by either Digitalis, Strophanthus or Squill; it is known that Strophanthus produces a comparatively small effect on the blood pressure, Apocynum causes a more immediate and sudden rise of blood pressure than Digitalis or Squill. There is no special difference in the action of these drugs on the kidney, which in each case is an indirect one, but Apocynum appears more likely to produce haematuria owing to its pronounced irritant properties.

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THE PHYSIOLOGICAL EFFECTS OF SELENIUM COMPOUNDS WITH RELATION TO THEIR ACTION ON GLYCOGEN AND SUGAR DERIVATIVES IN THE TISSUES

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Historical.

Action on Plants and Bacteria. The earliest studies on plants and bacteria were made by Knop in 1885. He found that on adding traces of selenium and tellurium salts to the water in which plants grew, that although no influence on the growth of the plants took place, yet selenium was absorbed. The same was found true of algae and infusoria by Bokorny in 1893. Scheurlen, in 1900, seeking a substance which contained loosely-bound oxygen, to grow bacteria in absence of atmospheric oxygen, tried sodium selenite, and found that though the bacteria were unaffected, yet they were coloured with the reduced selenium. This selenium was found entirely in the cell, none being found in the media. A careful study of these effects was conducted by Klett, who found that bacteria and moulds were not as a rule hindered in their development by traces of selenite of sodium, but a few, such as the bacillus of malignant oedema and symptomatic anthrax, were arrested in growth. He also found the bacteria coloured with the reduced selenium, the surrounding media being colourless, and that as the amount of selenite was increased growth was inhibited. He concluded that the reduction of selenite to selenium took place in the protoplasm of the bacterial cell, and not outside the cell by secondary action of metabolic products.

Action on animals. Gmelin appears to be the first who investigated the effects of selenium and tellurium salts on animals. He found that they were poisonous, that they produced a deposit of the reduced element on the intestinal walls, and that the animal gave off a garlic-like odour. This odour was also noticed in the animal's breath by Hansen, who attributed it to ethyl selenide; this observer found also that after a large

dose the animal vomited, and that the vomit contained selenium. On making sections of the animal's organs he noticed that they all contained selenium deposited in granules. The work was continued by Rabuteau, who observed that after a large dose vomiting, profound dyspnoea, anasthesia, opesthotonos, and death from asphyxia took place. The post-mortem findings were intense congestion and ecchymosis of the whole intestinal tract, also of the liver, spleen, lungs, and kidney. The right side of the heart and large blood vessels held a multitude of small prismatic crystals of unknown chemical composition. Rabuteau concluded that these crystals acted as a mechanical obstruction and caused death. These results were not confirmed by Czapek and Weil, who could not find any crystals or mechanical obstruction, and concluded that selenium was very similar in its action to tellurium, arsenic and antimony, and that death was due to paralysis of the so-called excito-motor ganglia. They further noticed marked distension of the abdominal capillaries. The blood was normal, but it gave off a marked garlic-like odour. This odour was noticed by Wöhler to be similar to methyl selenide, which he was then preparing. Hofmeister confirmed this, proving by analysis that they were the same, and further showed that all the organs gave off the odour, but that it was most pronounced in the testes and lungs, and marked in the blood, liver and kidney. If the organs were placed in an incubator the smell was intensified, but blood loses the smell. Hofmeister concluded that all the organs could absorb selenium and form methyl selenide from it; lastly he discovered that the reduction to selenium and the formation om methyl selenide were independent of one another. On heating an organ to 55° C. the formation of methyl selenide ceases, but the organ will still reduce selenite to selenium. The explanation suggested was that the reduced selenium was slowly built up into a soluble compound in the alkaline blood and was changed in the lungs to methyl selenide. The methyl groups he supposed to be derived from cholin, creatinin, and other methyl-bearing substances. The effects of selenium and tellurium salts on metabolism were investigated by Mead and Gies, and Woodruff and Gies, who found that selenium salts had little or no effect on metabolism, but that the ether-soluble substance in the faeces was increased. This they attributed to diminished absorption. They also examined the vomit resulting from selenium salts, and found that there was complete absence of free hydrochloric acid, the pepsin was unaltered, and on addition of hydrochloric acid digestion proceeded at a normal rate. Ptyalin, on the other hand, was markedly affected by selenium salts.

The compounds of selenium are thus extremely toxic; but if the amount present is very small, the cells are able to reduce it, forming an inert substance, and can then continue their metabolic changes.

This research was undertaken to endeavour to find out how this reduction is accomplished and also to find out more exactly the cause of death in selenium poisoning.

Two compounds of selenium were used in this reasearch, viz., sodium selenite and sodium selenate, both obtained from Kahlbaum.

Sodium selenate is a comparatively stable salt, easily soluble in water and neutral in reaction.

The lethal dose of selenate for a moderate sized rat (about 80 grammes) was found to be 0·6 c.c. of 0·125 per cent. solution.

Selenite of sodium is an extremely unstable salt. It is stated by Mead and Gies to be reduced by all protoplasm, and they have seen reduction take place in contact with fresh meat. It is even said that reduction takes place in contact with all organic matter. If this is so, it is difficult to see how any can be absorbed if given by the mouth, so that in this research all doses were given hypodermically.

The preparation used was found to be acid in reaction, the acidity of 1 gramme being equal to 2·75 c.c. normal sulphuric acid. It is distinguished from selenate most easily by the insolubility of the selenites of copper, cobalt and nickel. Cobalt salts give a mauve precipitate visible 1 in 800 of water; copper salts give an apple-green precipitate visible 1 in 1,200 water; nickel salts give a green precipitate visible 1 in 1,600 water. Owing to its instability the selenite solution was always made fresh as required.

The lethal dose for a moderate sized rat was 0·4 c.c. of 0·125 per cent. solution. The lethal dose for a moderate sized rabbit was 0·5 c.c. of 2 per cent. solution. The lethal dose for a moderate sized cat was 1 c.c. of 2 per cent. solution.

From these results it appears that selenate of sodium is only two-thirds as toxic as selenite.

After a *small* dose no symptoms were observed, and even the appetite was unaffected. As the dose was increased and was just sub-lethal, it was observed that, after about ten minutes, the animal became restless; this was followed by movements of the mouth, tongue and nose. As there were now present a peculiar garlic-like odour in the breath, it is probable that these movements are due to stimulation of the nerves

of taste and smell. Very shortly afterwards retching and vomiting commenced. If the dose has not been too great, recovery soon takes place, no after effects being noticeable. If the dose be too great, the vomiting and retching continue and somnolence passes on to unconsciousness and death.

It may be mentioned, as death has been ascribed by previous workers to dyspnoea, that laboured breathing was seen in one case only. It was in this case due to excessive reduction of the salt to selenium and consequent embolism of the pulmonary vessels. The paralysis, convulsions and other symptoms noticed by former investigators were probably due to the same cause, and are in no way connected with death from chemical poisoning with selenium salts.

Macroscopic and Microscopic Changes in the Tissues

The macroscopic *post-mortem* changes were very few. The liver was usually soft and friable. All the organs gave off the garlic-like odour noticed in the breath. The right side of the heart was distended and full of clot. The splanchnic vessels were enormously dilated.

The microscopic changes were more pronounced, and were investigated as follows: -The tissues were immediately placed in formol, dehydrated with acetone, embedded in paraffin and stained with eosin and haematoxylin.

The most noticeable feature of all the sections was a golden-brown amorphous deposit found in almost every organ. It is chiefly found around the blood vessels and between the cells, but some is also to be seen inside the cells. It was suggested that this substance might be iron, but it gave none of the staining reactions for iron. On grinding up the organs with sand this substance could be extracted, and formed a brick-red deposit. This was found to be identical in every way with the amorphous form of selenium produced on reducing sodium selenite in the test-tube. It volatilized with heat, burning with a blue flame, and gave off the well-known horseradish smell of selenium.

This golden-brown deposit was found, if the dose was large, in almost every organ, the whole of the tissues being flooded with it, but this is not the cause of death, for if a just lethal dose is given there is no such flooding and death still takes place. Physiologically this deposit is inert, for if a small dose of a selenium salt is given and the animal killed some time afterwards, this deposit will be seen in the cells, which are evidently still capable in its presence of performing their metabolic changes without noticeable change.

The action of selenium salts on different isolated physiological systems prepared by the usual methods was next investigated, and they were found to be without action on (1) a muscle-nerve preparation, (2) isolated heart muscle, (3) nervous mechanism of heart, (4) higher nerve centres, (5) blood pressure, (6) intestinal movements. The urine was also normal, traces of selenium salts were found present, but never any solution that reduced Fehling's solution. The blood was occasionally found altered; the most frequent change was a slight lymphocytosis followed by a more marked increase in the polymorphonuclear leucocytes.

The red blood cells were found normal both in their number and in their haemoglobin contents, and spectroscopically the blood was found normal. In small amounts, selenate had no effect on gastric digestion, nor had it on pancreatic, while selenite had an inhibiting effect on pancreatic action, this being in part at least due to its acid reaction.

The reduction which takes place in the tissues was next investigated. After heating an organ to 60° C., as Hofmeister showed, it will still reduce selenite to selenium. This was repeated, and it was further seen that higher temperatures do not stop this reduction. It therefore seemed probable that this effect was not due to an enzyme, but was some direct chemical effect. A fresh solution of sodium selenite was therefore made, and 5 c.c. added to 0·5 gramme of each of the following carbohydrates with aseptic precautions. The mixtures were then placed in an incubator at 35° C. for twenty-four hours and again examined.

Reduction was shown by the solution becoming pale brown, while if the reduction were intense a fine brick-red powder became deposited.

POLYSACCHARIDES	Glycogen ...	No action
	Inulin ...	Profuse reduction
	Starch ...	No action
	Dextrin ...	No action
HEXATOMIC ALCOHOLS ...	Mannite ...	No action
	Dulcite ...	No action
PENTOSES	Arabinose ...	Reduction
	Rhamnose ...	No action
	Xylose ...	No action
HEXOSES	Maltose ...	Faint reduction
	Glucose ...	Reduction
	Galactose ...	Slight reduction
	Lactose ...	Slight reduction
	Levulose ...	Profuse reduction
AMINO HEXOSE	Glucosamine ...	Profuse reduction
COMPOUND SUGARS... ...	Saccharose ...	No action
	Raffinose ...	No action

The reduction of inulin is due to the formation of levulose.

It will be seen that reduction takes place with arabinose, levulose, glucose, and the sugars yielding glucose. The reduction by glucose and levulose was then tried at a low temperature, viz., 30° C., and it was found that glucose caused no reduction at this temperature even after several weeks, while levulose caused a profuse reduction. When the two sugars were mixed it was found that no reduction occurred at 30° C., and both came down at 38° C. It was thought that perhaps this reaction with more rapid heating might serve to differentiate the sugars, but on heating a solution of the sugars with sodium selenite solution in a test-tube, it was found that levulose commenced to reduce at 58° C., while glucose, lactose, galactose and maltose all reduced at about the same temperature, viz., 72° C.

The derivatives of glucose and its compound sugars were next tested as reducing agents for selenite, with the following results:—

Glucose	...	Reduction
Gluconic Acid	...	No action
Glucuronic acid	...	No action
Saccharic acid	...	Reduction
Mucic acid	...	No action
Furfurol	...	No action.

The above reductions at first sight would appear due to the aldehyde or ketone groups in the sugars, but here we are met with the fact that while rhamnose and xylose, which contain aldehyde groups, have no action, yet saccharic acid, which has neither aldehyde nor ketone group, acts very strongly. We tried the action of benzaldehyde, formaldehyde and acetone, but found they all gave negative results.

Other possible degradation products of the sugars were tried, for example, lactic acid and acetic acid were without action, but formic acid had a powerful reducing action.

It is interesting to note here that selenite is reduced by arabinose, levulose, glucose, maltose and lactose, all of which are found in, or are excreted from, the human body. The only exception is xylose, which is a necessary constituent of the nucleoproteids, but in such a position an energetic sugar would be a source of danger to the animal. These results cannot be explained on the structural formulae at present ascribed to the different sugars, so that the reduction rests more on a physiological basis than a purely chemical one. It was next ascertained whether proteins, fats, or other substances of animal origin, would perform the same

reduction. Although treated in the same way as the carbohydrate or heated together directly in the test-tube, no action was found associated with any one of them. The following substances were all tried, viz.:—Olein, oleic acid, palmitin, palmitic acid, potassium palmitate, erucic acid, lecithin, cholesterin, glycerine, uric acid, hippuric acid, urea, gelatin, glycocoll, tyrosin, casein and creatinin.

From this it appears probable that this reduction can be performed by glucose and a few allied sugars, and cannot be produced by organic matter from which carbohydrates are absent.

To ascertain whether glucose is the agent which accomplishes this reduction in animal organs, the following method was adopted. Ten grammes of finely minced liver were taken with aseptic precautions, and to this were added 5 grammes of yeast, 50 c.c. of distilled water and a few drops of toluol. A control was prepared in the same way, *but without any yeast*. Both vessels were placed in an incubator for twenty-four hours, they were then heated to 70° C. to destroy the yeast and glycolytic enzyme, and 1 gramme of sodium selenite added to each. After being in an incubator at 38° C. for a few hours, both were examined. The control which contained glucose from the glycogen was of a deep red colour, showing that quantities of selenium were present. The flask which had its sugar destroyed by the yeast showed no red colour, so that no selenite had been reduced. This experiment makes it probable that in the absence of glucose selenite is not reduced, and, accordingly, that reduction of selenite to selenium in a cell indicates the presence of glucose.

Since selenite of sodium is reduced in the cells by glucose, it appeared necessary to ascertain where, especially in the body, this reduction takes place. As has been already seen, if a large dose is given the whole organism is flooded with the selenium. A rat was therefore given several *small* doses, and was then killed, and the organs quickly removed and examined histologically.

The spleen was found to contain abundance of selenium, as well as an excessive number of leucocytes.

The portal vein was examined and found to also contain selenium and leucocytes, and the same was found true of the liver; while the vessels leaving the liver, the lungs, kidney and intestine, were found to be quite free from selenium.

In some cases the liver cells show destructive changes. The nucleus

stains less deeply while in others the nucleus has disappeared, the cells being mere shadows.

It therefore seems probable that reduction takes place firstly in the spleen. The reduced selenium is brought by the blood stream, only a small amount by leucocytes to the liver. The liver also reduces any selenite that has escaped the spleen. If the dose is not excessive no selenium is allowed to pass the liver.

Disappearance of Glycogen from the Liver

Finding that selenite is reduced by glucose in the liver and spleen suggests that the glucose must be derived from the liver glycogen, and this was found to be the case. Two well-fed rats were taken, and to one was given an injection of sodium selenite just sublethal. As soon as it began to recover, which happened in a few hours, another injection was given. Treated in this way, in a time varying from three to seven days, the animal dies. The control rat was then killed, and the livers from the two animals contrasted as to their glycogen content by the following method:—The livers from both rats were quickly removed, cut in pieces in each case, and placed separately in boiling water acidified with acetic acid. The pieces were then ground up in each case in a mortar with hot distilled water.

In the rat which had been injected with selenite, there resulted a perfectly clear pale orange coloured solution, which gave no coloration with iodine and no precipitate with alcohol or basic lead acetate.

The fluid from the normal rat's liver gave an opalescent solution, which on addition of iodine gave a dense brown coloration.

This experiment, on account of its importance, was repeated several times, with the same result. It may be stated that the dose given must be carefully regulated so as to be just sublethal. If an over-dose is given, the animal will die, due probably to not sufficient glucose being available to reduce the selenite; glycogen will then not have disappeared entirely. This disappearance of glycogen was also found to occur in both frogs and rabbits.

This gradual using up of glycogen and glucose made it interesting to find out if any other metabolic changes took place at the same time.

A well-nourished cat was used for the experiment. The normal excretions were examined and estimated daily for a week, during which period the urine was invariably found to be acid. A small injection was

then made of 0·5 c.c. of 0·25 per cent. solution of selenite; the only change following this small dose that took place was that the urine next day had become alkaline; this continued so throughout the experiment. The dose was gradually increased, and it was noticed that the amount of urea excreted on the day following the injection had fallen considerably, but had returned to normal on the following day. As the dose increased, the urea decrement became greater, and an increased number of days were required for the return to the normal. When the dose became excessive the animal vomited. No urine was excreted the following day. The next day the excretion of urea was exceedingly small, and continued small for some days, only very slowly returning to the normal. The total amount of nitrogen excreted showed no relation to the urea; it kept steady all through the periods when the urea fell. When an excessive dose was reached the total nitrogen fell to about one-third the normal amount and gradually returned to normal.

It would thus appear that while the urea was excreted in less amount the nitrogen was got rid of in some other form. That it was not excreted as uric acid nor as ammonia seems probable as these showed little or no change throughout the experiment. Sulphates and phosphates showed no change.

The most striking effect was noticed in the excretion of chlorides. Until the dose had become excessive the amount of chlorides thrown out had continued steady. After the animal vomited the excretion of chlorides suddenly dropped, so that the daily amount excreted became half or less than half the normal amount; thus, in the first cat the average daily output was 1·28 grammes; this fell to 0·061 grammes.

In the second cat, as will be seen in the following table, the average daily excretion of chlorides for fifteen days during which it was having increasing amounts of selenite was 0·0749 grammes. After 0·75 c.c. of 2 per cent. solution had been injected the animal vomited. No urine was passed the following day. The next day the amount was only 0·0305 grammes, that is, less than half the normal amount. The following three days there was very little increase. The following day, as will be seen in the table, the whole of the retained chlorides were thrown out, the amount excreted afterwards returning to normal again.

Chlorides excreted per diem in grams.								
0·0749	Injection	Vomited No urine	0·0305	0·0335	0·0381	0·0441	**0·1820**	0·0611

The vomiting took place about ten minutes after injection. The vomit consisted of the stomach contents in a state which showed that there had been no interference with digestion until the injection was given. The animal during this period was under the influence of the drug, and it is only when it reaches an excessive amount that vomiting occurs. This vomit was found, as already pointed out by Mead and Gies, to be free from any trace of free hydrochloric acid. The reaction was acid, and quantities of organic acid were present.

It therefore seems probable that the hydrochloric acid is suddenly withdrawn from the stomach to serve some other necessary purpose, and if 0·2 per cent. hydrochloric acid be added to the stomach contents digestion will proceed normally.

This withdrawal of hydrochloric acid is accompanied by a greatly diminished excretion of chlorides in the urine. This observation coincides with the withdrawal of hydrochloric acid from the stomach. While the chlorides are retained, appetite and digestion are in abeyance; after a certain period, varying from one day to five days, during which time the excretion of chlorides is only about half the normal amount, the whole of the chlorides retained are thrown out, their purpose having been fulfilled. Then the animal regains its appetite and the excretion of chlorides becomes normal.

Still another interesting fact bearing on the subject was noted: when the animal had lost its appetite, although it refused fresh meat it would still eat salt meat. Possibly the excess of chloride helped the return of hydrochloric acid.

Lastly, with these changes there was also a remarkable loss of weight. One cat lost 38 per cent. of its total weight, the average daily loss being 26 grammes. A second cat lost 335 grammes during the first week, or 16 per cent. of its weight, and in one day it even lost as much as 65 grammes. This loss of weight is too great to be accounted for by diminished consumption of food alone, for until the dosage became large the animal still retained its appetite, and even after a large dose it only refused its food for a day or two, the appetite gradually returning.

It was observed by Mead and Gies that after a dose of a selenium compound the amount of ether-soluble substance in the faeces was increased. This we found true for small doses, but on investigating the effect of large doses we found that the amount of ether-soluble matter in the faeces was very much diminished, and that the relative amounts of neutral fat, fatty acid and soap (reckoned as oleic acid) were altered.

The faeces were dried and extracted with ether in a Soxhlet apparatus, the acidity of the fat being titrated with 0·1 N alcoholic potash, using phenol phthalein as indicator. After an injection of selenite of sodium the total amount of fat in the faeces became very much reduced and was at its minimum the second day after injection. The excretion of fatty acid was less affected or increased in amount, so that the normal proportion of neutral fat to fatty acid being 2 to 1 on the second day, the fatty acid became equal to, and sometimes greater than, the amount of neutral fat. This may be seen from the following table, where the amount of fat has been worked out to a constant:

Fat		Free fatty acid
0·5	...	0·284
0·5	...	0·292
0·5	...	0·290
	Injection of selenite	
0·5	...	0·350
0·5	...	0·530
0·5	...	0·170
0·5	...	0·280

The effect on soaps is somewhat similar to the fatty acid, the excretion being greatest the second day after an injection and gradually falling from then onward.

These observations seem to show that glucose, or a derivative, is the means by which the body protects itself from the toxic effects of selenium salts, and death may even in certain cases be due indirectly to the using up of the glucose; but this cannot be the cause of death after a single large dose, therefore the effect of selenite on the living cells of the liver was next investigated. A fresh liver was finely minced, using aseptic precautions, and 10 grammes were weighed out in every case for the purposes of the experiment. To this were added 50 c.c. of sterile distilled water and a few drops of toluol. The substance to be tested was added to one, and the control and the one containing selenite were placed in an incubator at 38° C. for varying periods of time. They were then boiled and filtered. The filtrate and solid matter were each separately estimated by Kjeldahl's method.

SELENATE OF SODIUM

		Soluble Nitrogen		Insoluble Nitrogen
Normal	... ...	0·0883	...	0·2125
Selenate 0·5 gramme	...	0·1076	...	0·1794
Normal	... ...	0·1674	...	0·1618
Selenate ½ %	... ...	0·1268	...	0·2047
Selenate 1 %	... ...	0·1570	...	0·1751

We see that there is no definite effect on autolysis caused by selenate of sodium.

SELENITE OF SODIUM

Third day

	Soluble Nitrogen	Insoluble Nitrogen
Normal boiled	0·0192 ...	0·2711
Selenite 0·5 gramme ...	0·0211 ...	0·2708
Normal liver	0·0883 ...	0·2123

Seventh day

	Soluble Nitrogen	Insoluble Nitrogen
Normal liver	0·1911 ...	0·1497
Selenite 0·5 grammes ...	0·0762 ...	0·2636
Selenite 1 gramme	0·0566 ...	0·2763

Tenth day

	Soluble Nitrogen	Insoluble Nitrogen
Normal liver	0·1674 ...	0·1616
Selenite 0·5 grammes ...	0·0636 ...	0·1171
Selenite 2 grammes	0·0745 ...	0·1047

It is evident here that selenite of sodium has a very marked inhibitory effect on autolysis. The presence of selenite in a cell in sufficient amount would seem to inhibit all metabolic changes and destroy the cell. As the action of selenate of sodium in the body is similar to selenite in its ulterior effects, and as selenate is not poisonous to the cells, it seems evident that its toxic effects are present only when it has been reduced in the body to selenite.

The ease with which the glucose molecule is broken up by sodium selenite and selenate in the body suggested that if *diabetes mellitus* were due to the inability of the animal cells to break up the glucose molecule, as the believers in the oxidation theory hold, then the presence of selenite or selenate which effects this splitting up should lessen the amount of sugar in the urine, for it is well known that the degradation products of glucose can be easily dealt with by the diabetic. Selenate of sodium was the salt used, being less easily reduced in the intestine than the selenite. The patient was under the care of Dr. J. Hill Abram, whom I have to thank for trying the drug, and also Dr. A. F. Jackson for the care with which he performed the sugar estimations. The patient was a case of severe diabetes with twelve months' history.

	Per diem	
	Average sugar, in grains	Urea, in grains
Common diet	5232 ...	538
Special diet	2212 ...	307
Selenate	3087 ...	455

The dose of selenate given was 5 minims of 1 per cent. solution, gradually increased until 25 minims was given.

As will be seen, there was an actual increase in excreted sugar while taking selenate, which seems to show that diabetes is not due to any lack of oxidation power nor to any difficulty in breaking up the glucose molecule.

Conclusions

These observations make it probable that selenate is reduced in the body to selenite, so that the action of selenite only need be considered.

When an injection of selenite is given, it is quickly taken up by the blood stream; only a small quantity is excreted by the kidney, the remainder is carried to the spleen and liver, where it is reduced by glucose to selenium. According to Zsigmondy, this reduction with glucose can, with the aid of the ultra microscope, be seen to take place fairly easily outside the body, particles of the reduced selenium being visible in about two minutes, so that the living cells would have no difficulty in effecting the same.

This reduction does not appear due to the aldehyde group of the sugar, but more probably is due to some special configuration of the glucose molecule, in which it is closely resembled by arabinose and levolose. This glucose, as required, is furnished from the glycogen of the liver, but when this is becoming exhausted fat is called upon. Whether the fat is used up as such or is transferred into sugar, it is difficult to say. The excretion of excess of fatty acid after an injection when sugar is urgently required, would point to the glycerine being possibly required for transference to sugar, but it is clear that there is no effort on the part of the organism to form sugar from proteins. If it were possible an effort would be made by the cells to manufacture glucose from protein to save themselves from destruction, but that no such action occurs is shown by the excretion of nitrogen remaining low, even until death. One must conclude that the organism cannot under such conditions transfer protein into sugar.

The evidence as to the transference or at least equivalence of fat and sugar is better. There is complete disappearance of fat as well as glycogen and sugar, which points to their transference or utilization. The loss of weight also suggests the same. In a moderately fat cat the loss of weight was about 38 per cent. This would just about represent the weight of fat, glycogen and glucose. Möchel's estimate of fat in a

moderately fat dog being 26 per cent., the remaining 12 per cent. would account for the glycogen and glucose.

It is necessary to consider at this stage the sudden disappearance of hydrochloric acid, the holding back of the chlorides by the organism, and the extraordinary relish for sodium chloride. These factors are all present when selenite has just been given, and when there is a sudden demand for glucose on the part of the cells. The most likely explanation is to be found in an observation by Eckhard that a one per cent. solution of common salt introduced into the blood caused glycosuria. Fisher showed that other sodium salts had the same effect, and that the stronger the salt solution the more glycosuria resulted, even up to 7·3 per cent. of sugar was found.

Excess of sugar in the urine represents excess of sugar in the blood, and therefore it would be possible for an animal holding back its chlorides by using its hydrochloric acid to fix some sodium salt, and taking sodium chloride in its food to raise its blood sugar content. If this sugar is picked out by the spleen and liver, and also possibly by leucocytes, it must result in a wonderfully increased power of reducing selenite to selenium, and so saving the cells from the poisoning effect.

If the organism is unable to neutralize the selenite, either because the selenite is in too great excess or because the available stores of glucose are used up, then selenite will act on and destroy the cells. The action is on the ferments of the cells, causing all metabolic changes to cease. It is curious to note here what a slight effect it has on bacteria and a powerful effect on ferments, quite a contrary effect to toluol, chloroform and similar antiseptics. It seems to show that the defences in single cells are much more highly developed than is the case in cells which rely on others for protection.

It is interesting to consider here whether glucose may not possibly be the means by which all reduction processes take place in the body. The well-known reductions taking place in the organism, such as methylene blue and Prussian blue, can be accomplished with ease by glucose in faintly alkaline solution. Considering the universality of occurrence of glucose in the body cells, and its well-known power of reduction outside the body, which is immensely multiplied within by cell activity, it would hardly seem necessary for the cells to require any other means of reduction.

I take this opportunity of expressing my indebtedness to Professor Benjamin Moore and Professor C. S. Sherrington for their kind assistance and advice.

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THE EFFECT OF WORK ON THE CREATINE CONTENT OF MUSCLE

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Previous Work

The question—Does work influence the amount of creatine in muscle, and lead to a change in the output of creatinine in the urine, has long been a debated one. Much of the older work on this point is of little value owing to the defective methods then available for the estimation of creatine and creatinine. The papers of Weber (1) and van Hoogenhuyze and Verploegh (2) give good accounts of these earlier investigations.

Of the older workers, Liebig (3) found an increase of creatine in muscle after work; as did also Sarokow (4), Sczelkow (5), and Monari (6). Nawrocki (7), and Voit (8) thought that there was a slight decrease. Of modern workers Weber (*loc. cit.*), using Folin's method, found that work caused a slight decrease in the creatine content of muscle. He also found that traces of creatine and creatinine could be demonstrated in the Ringer's fluid with which, using the method of Langendorff, he had perfused an isolated heart; while Mellanby (9), also using Folin's method, obtained results from which he concluded that work had no influence on the creatine content of muscle.

As regards the effect of work on the excretion of creatinine in the urine there is also a difference of opinion. Meissner (10), employing very defective methods, found an increase of creatinine in the urine on the day of exercise, followed by a decrease on the succeeding day. Grocco (11), and Moitessier (12) also found an increased output on the day of exercise. Gregor (13) concluded from personal experiment, using a creatinine free diet, that exercise increased the output of creatinine in the urine. Voit (*loc. cit.*), Hoffmann (14), Oddi and Tarulli (15), using the older methods found no increase. Van Hoogenhuyze and Verploegh (*loc. cit.*), using Folin's method, found that, with an ample diet—creatinine free, work left the excretion of creatinine unaffected, but that when the diet was defective, as during the complete fast of their subject, La Tosca, exercise resulted in a slight increase of creatinine in the urine. Weber

(*loc. cit.*) also came to the conclusion that, if work were performed during a fast, a rise in the output of the creatinine followed. Shaffer (16) agreed with the results of van Hoogenhuyze and Verploegh. One of us (E. P. C.), although not investigating this particular point, working in conjunction with Drs. Kennaway and Leathes (17), was unable to detect any definite rise in the output of creatinine even after severe work under very different conditions, in all of which, however, the diet was ample. We may therefore accept that, provided the supply of food is sufficient, work does not bring about any increase of creatinine in the urine.

Present Investigation

As regards the results on stimulating frog muscle we have already made a communication (18), in which we showed that there is an increase in the amount of total creatinine in stimulated isolated frog muscle (ordinary nerve muscle preparation), whereas, when the circulation is left intact as in the decerebrated frog, there is always a slight decrease in the amount of the total creatinine present.

The following table gives the results with frog muscle:—

Table I

Series A. (Isolated nerve muscle preparation.)

Experiment	Per cent of total creatinine in normal (controls)	Per cent. of total creatinine in stimulated	Difference
A	0·32	0·36	12 % increase
B	0·30	0·32	7 % ,,
C	0·32	0·36	13 % ,,
D	0·36	0·39	8 % ,,

Series B. (Muscle *in situ*, circulation intact.)

Experiment	Per cent of total creatinine in normal (controls)	Per cent. of total creatinine in stimulated	Difference
A	0·37	0·26	15·6 % decrease
B	0·35	0·29	6·2 % ,,
C	0·30	0·23	9·1 % ,,
D	0·32	0·24	7·7 % ,,

We have now carried out a series of experiments on rabbit muscle under different conditions, and have obtained constant results.

The methods we employed were as follows:—The rabbit was deeply anaesthetised with ether and was kept under the influence of the anaesthetic till the end of the experiment, when it was destroyed. The extensor muscles of the right knee were exposed by means of a skin incision and removed, the amount taken being usually 10 to 15 grammes. The

bleeding points were secured and care was taken not to injure the great vessels of the thigh. All visible fatty and fibrous tissue, as well as blood, was rapidly removed from the excised mass, which was then weighed, transferred to a small mortar, minced fine by means of a pair of sharp scissors, then rubbed up with finely powdered glass, water being gradually added until a fine suspension was obtained. This mixture was next carefully transferred to an Erlenmeyer flask, filled up to 150 c.c. with distilled water, some chloroform and thymol solution added, thoroughly shaken up, and then placed in a hot water oven at 50° C., where it was left, being repeatedly shaken, for eight to ten hours. At the end of this period the flask, after faintly acidifying its contents with acetic acid, was put on the steam bath, or boiling water bath, for thirty minutes and then filtered. The solid residue was extracted with boiling water (as a rule by boiling the residue with water in a porcelain basin) five to seven times and filtered through the original filter. The united filtrates were then concentrated to 40 c.c., and the content of creatine + creatinine estimated by Folin's colorimetric method. The same procedure was carried out in the case of the stimulated extensor muscles of the left thigh, care being taken (i) that the corresponding muscles were taken for examination, and (ii) that the amount taken differed by several grammes from that taken from the right (unstimulated) side—sometimes more being taken, sometimes less—so that the readings on the colorimeter were widely apart, thus obviating to a considerable extent any possible personal bias. As an additional precaution one of the observers frequently manipulated the solutions in such a manner that the observer who took the readings was unaware which of the two solutions he was examining. The reason why definite corresponding muscles were chosen was that as the result of several experiments we carried out with different kinds of muscle (red and white) we are inclined to believe that there is some difference in their creatine content. That such might be the case is suggested by such work as that of Bonhöffer (19) and of Paukul (20), who showed that in muscle structural differences might be associated with functional. So far we have not performed a sufficient number of experiments on this point to be able to draw definite conclusions.

As regards our method of stimulation, immediately after the removal of the extensor muscles of the right side those of the left were transfixed by a pair of electrodes, one of which passed through the muscles close to the patella and the other near their origin. Through these electrodes the muscles were stimulated with rapid alternating faradic shocks. A

Berne coil was used, the primary current being about 5 volts and the secondary coil was placed between the 2,000 and 4,000 unit marks. The muscles were stimulated over a period of thirty to forty-five minutes, but the secondary currents were not allowed to pass continuously through them, being short-circuited for five seconds in every ten seconds.

We carried out control experiments to determine the accuracy of our chemical methods, and found that, for small amounts of muscle, the average difference between the extensors of the two sides, neither having been stimulated, was a little over 1 per cent. of the total creatinine, and for larger amounts (10 to 15 grammes) was about 2·3 per cent.

The general result of the whole series of experiments has been that, with the circulation intact, stimulation of the muscles brings about a constant, although small, decrease in the amount of total creatinine (i.e., creatine + creatinine) extracted from the stimulated muscle.

Experiment II.—Rabbit, weight 1,550 grams. 12 grams muscle removed before stimulation (stimulation 30 minutes) and 11 grams after. Total creatinine before stimulation, 43·42 milligrams = 0·361 %, and after, 30·84 milligrams = 0·280 %, i.e. a decrease of 22·4 % as a result of stimulation.

Experiment IV.—Rabbit, weight 1,850 grams. 7·5 grams muscle removed before and 17 grams after 40 minutes' stimulation. During the period of anaesthesia a free supply of pure oxygen was given. Total creatinine before stimulation 28·8 milligrams = 0·334 %, after stimulation 54·6 milligrams = 0·321 %, i.e. a decrease of 4·3 % as a result of stimulation.

Experiment V.—Rabbit, weight 1,450 grams. 9·2 grams muscle removed before and 16 grams after 30 minutes' stimulation. Total creatinine obtained before, 37·84 milligrams = 0·411 %, and 52·4 milligrams after = 0·327 %, i.e. a decrease of 20·4 %.

Experiment VI.—Rabbit, weight 2,200 grams. 10 grams muscle removed before and 12·5 grams after 30 minutes' stimulation. A free supply of pure oxygen was allowed during the period of anaesthesia. Total creatinine before stimulation 30·60 milligrams = 0·306 %, and 37·68 milligrams after = 0·301 %, i.e. a decrease of 1·6 %.

Experiment IX.—Rabbit, weight 2,250 grams. 9·1 grams muscle removed before and 14·2 grams after 35 minutes' stimulation. Total creatinine in muscle, before stimulation 39·04 milligrams = 0·429 %, and 51·42 milligrams after = 0·361 %, i.e. a decrease of 15·8 %.

Experiment X.—Rabbit, weight 1,900 grams. 11·5 grams muscle removed before, and 14·8 grams after 35 minutes' stimulation. Total creatinine in muscle, before 43·24 milligrams = 0·375 %, and 50 milligrams after = 0·337 %, i.e. a decrease of 10·1 %.

As an example of two control experiments done, the following may be given, No. 7 with our maximum error, and No. 12 with our minimum.

Experiment VII.—Rabbit. 15 grams of muscle removed from right, and 13·1 grams from left thigh. Total creatinine, in muscle from right side 53·08 milligrams = 0·354 %, and from left side 48·0 milligrams = 0·366 %, i.e. a difference of 3·3 %.

Experiment XII.—Rabbit. 8 grams of muscle removed from right thigh and 11 grams from left. Total creatinine, in muscle of right side 36·93 milligrams = 0·461 %, and from left side 50·31 milligrams = 0·457 %, i.e. a difference of 0·86 %.

If Mellanby's figures be examined, although he draws the conclusion from them that work is without influence on the creatine content of muscle, it will be noted (*loc. cit.* pp. 459-460) that his frog's muscle stimulated when isolated always show a slight increase in amount of total creatinine present, perhaps somewhat smaller than ours, and, in the two experiments on rabbits, which he did with their circulation intact, there is in both cases a slight decrease.

As already mentioned, van Hoogenhuyze and Verploegh have come to the conclusion, in which they are supported by other investigators, that work does not increase the output of creatinine in the urine when the diet is sufficient.

It was thought that the state of the nutrition of the animal, or, perhaps, variations in the carbohydrate reserve, might influence the total creatinine content of worked muscle. The experiments given above may be divided into two series, one in which the animals were kept on a very low cabbage diet for four or five days preceding the experiment, and the other in which the animals were allowed to feed freely on bran, turnip, and carrot for the same length of time. In every case there was a decrease in total creatinine, but the decrease was somewhat greater in the badly fed than in the well fed animals.

Table II.

Experiment		Total creatinine in milligrams	Per cent. present in muscle	Decrease per cent.	Mean decrease per cent.
	Series A. Badly fed				
2.	B(efore)	43·42	0·361	22·4	
	A(fter)	30·84	0·280		
5.	B.	37·84	0·411	20·4	
	A	52·40	0·327		
10.	B	43·24	0·375	10·1	
	A.	50·00	0·337		
				52·9	17·63
	Series B. Well fed				
4.	B.	28·8	0·334	4·3	
	A.	54·6	0·321		
6.	B.	30·60	0·306	1·6	
	A.	37·68	0·301		
9.	B.	39·04	0·429	15·8	
	A.	51·24	0·361		
				21·7	7·23

12.16
3.67
9

7416

Other experiments with frogs were carried out along these same lines, previous to the rabbit experiments. In these the frogs were given large quantities of glucose for two days before stimulation, but the results which we obtained by this method were not very concordant.

Another point which appeared in the course of our work was the constant gain in fluid which occurred in the muscle as a result of stimulation. We found that, in the muscles of both frogs and rabbits, the average gain after thirty to forty minutes' stimulation was about 2 per cent. We discovered subsequently that this figure was almost identical with that given by Ranke (21). In our experiments the fresh muscle was weighed as soon as possible after removal, then dried at 100° C. till constant weight was obtained.

In every experiment, although our method is not calculated to elucidate the question, we examined the muscle extract for preformed(?) creatinine, but found that the amounts present varied very largely in quantity, the variation being also found in the control experiments. We are inclined to agree with Mellanby that muscle contains no preformed creatinine, or at most mere traces of this substance. The variation in the amounts found in our experiments were due in all probability to a partial conversion of creatine to creatinine during the process of extraction. There was, however, one interesting fact which may be mentioned in this connection, and that was that the percentage amount of preformed (?) creatinine present in the above experiments with rabbit muscle showed, with the exception of one experiment, a decrease when there was a decrease of total creatinine.

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THE ACTION OF EXTRACTS OF THE PITUITARY BODY

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I. Introductory

Though the activity of pituitary extracts was discovered by Oliver and Schäfer (1) almost simultaneously with that of suprarenal extracts, the conceptions of the nature of the action of the former are as yet far less precise. A comparison of the two was inevitable, and it has more than once been suggested that their action, at least as regards vaso-constriction, is of the same kind and produced by stimulation of the same structures. Herring (2) advanced this view as regards the arteries: a more recent observation by Cramer (3), of the action of pituitary extract on the pupil of the frog's eye (enucleated), lends support to the same idea: still more recently an account given by Bell and Hick (4) of the action on the uterus emphasised the similarity between the action of extracts from the two organs. I thought it worth while, therefore, to bring together a number of observations, made at different times and in different connexions, which appear to me to indicate that such correspondence as exists is wholly superficial and illusory. In the first place it must be admitted that the actions of pituitary and suprarenal extracts have superficially several points of suggestive similarity. Both raise the blood-pressure, peripheral vaso-constriction being a principal factor in the effect (Oliver and Schäfer): in both cases the active principle is limited to a small, morphologically independent portion of the gland, developmentally related to the central nervous system in the one case, as to the sympathetic system in the other. Attention is drawn to these points of similarity by Schäfer and Herring (5), who state that 'here the parallelism ends': but the divergence of which they make specific mention is that the pituitary extract has an additional effect on the kidney. Since they attribute this to a separate active principle, no true divergence is indicated between the *pressor* principles of the two organs. It has been shown (Langley (6), Brodie and Dixon (7), Elliott (8)) that the action of adrenaline reproduces with striking accuracy the effects of stimulating nerves of the true

sympathetic or thoracico-lumbar division of the autonomic system. An examination of the action of pituitary extract on various organs and systems containing plain muscle and gland-cells will indicate whether its action has more than a superficial resemblance to that of adrenaline by showing whether its effects, or any group of them, can be similarly summarised by relating them to a particular element of the visceral nervous system. Incidentally evidence will be discussed which throws light on the contention of Schäfer and Herring that two active principles exist in the extract, one acting on the circulatory system, the other specifically on the kidney.

The extract used in my experiments, except where otherwise stated, was a 5 per cent. decoction of the fresh posterior lobes of ox pituitaries. The posterior lobes were dissected clean from the rest of the gland and from dura water, weighed in the moist condition, pounded with sand, and boiled with water faintly acidulated with acetic acid to produce coagulation. The exfract, filtered from coagulum, is a clear colourless fluid giving a faint biuret reaction. For experiments on isolated organs the extract was prepared with Ringer's solution and carefully neutralised before use.

II. The Effect on the Circulatory System

It has been mentioned that pituitary extract causes a striking rise of blood-pressure, chiefly due to arterial constriction. If the action had any relation to innervation by the sympathetic system we should expect to find that the effect on the arteries was accompanied by an increased frequency and force of the heart-beat, corresponding to the effect of the cardio-accelerator nerves. It was pointed out by Schäfer and Oliver that this was not the case: the beat of the heart usually becomes slower, even after exclusion of vagus action, though it may be somewhat augmented. Reference will be made later to the action of the extract on the isolated heart, which enables the effect to be studied in its least complicated form.

We should further expect to find, if the action were like that produced by sympathetic nerve-impulses, that the action on the arteries showed irregularities of distribution corresponding to that of sympathetic nerves. It was of special interest, therefore, to examine the action on those arteries which have been shown to be exceptional in their innervation and in their reaction to adrenaline.

The pulmonary arteries. Brodie and Dixon showed that the peripheral branches of the pulmonary artery are exceptional in that their

muscular coats are not under the control of sympathetic nerves, and made the interesting parallel observation that adrenaline, perfused through the pulmonary vessels, produces no vaso-constrictor but a small vaso-dilator effect. With segments of the main branches of the pulmonary artery, treated as isolated organs, others have obtained definite constrictor effects with adrenaline (Meyer (9), Langendorff (10)). It is clear that there is no real discrepancy between the two sets of observations: the only conclusion justified by the evidence is that the sympathetic nerves send motor fibres to the muscular walls of the pulmonary artery and its main branches, but that the innervation stops short of the peripheral arterioles, the calibre of which is alone concerned in determining the rate of perfusion under constant pressure, as measured by Brodie and Dixon.

In a few experiments with isolated rings of large branches of the pulmonary arteries of large dogs and goats, I observed contraction on adding small quantities of the pituitary extract to the Ringer's solution in which the rings were suspended. Since these experiments were made similar observations have been published by de Bonis and Susanna (11). Since, however, I obtained even more pronounced constriction of the strips of pulmonary artery on adding adrenaline, these results only add another to the cases already known in which adrenaline and pituitary extract both cause constriction of an artery, and are of no significance for our present enquiry. I owe to Professor Dixon the opportunity of making with him observations on the effect of pituitary extract on the peripheral pulmonary arterioles. The observations were made in connéction with experiments concerning action on these arterioles of certain organic bases. The lungs were perfused with Ringer's solution, or defibrinated blood diluted therewith, according to the method described by Brodie and Dixon. After it had been shown that either adrenaline or p. hydroxyphenyl-ethylamine caused only a slight acceleration of the rate of perfusion, 1 c.c. of the pituitary extract was introduced into the circulating fluid. As soon as the extract reached the lungs there was a pronounced retardation of the outflow. The observation was repeated several times, in different experiments, with uniform result. Here, then, is a clear case of vaso-constriction produced by pituitary extract on a system in which no such constriction is produced by adrenaline or substances of similar action.

The coronary arteries. The innervation of the coronary arteries cannot be regarded yet as definitely settled, even the more recent observations being by no means concordant. Maas (12) found that the vagus supplies vaso-constrictor fibres to this system: Dogiel and

Archangelsky (13) found that vaso-constrictor fibres are contained in the accelerator nerves: on the other hand Schäfer (14) could not find any evidence for vaso-motor nerves to these arteries, and observed no constriction of them under the influence of adrenaline. The last observation was confirmed by Elliott (8), who found the outflow from a perfused segment of ventricle increased by adrenaline. Langendorff observed that adrenaline caused relaxation of an isolated ring of coronary artery, and this has been confirmed by de Bonis and Susanna. Still more recently Wiggers (15) has found evidence of vaso-constriction when adrenaline is added to a fluid perfusing the coronary arteries. From all this conflicting evidence emerge the facts that the coronary arteries are slightly, if at all, controlled by vaso-motor nerves, and that the constrictor effect of adrenaline on the peripheral branches, if it exist at all, is very weak compared with the effect of that principle on other arteries.

In this instance I made no experiments with isolated rings of artery, but such have recently been published by Pal (16) and by de Bonis and Susanna. These observers agree in finding that pituitary extract causes a marked constriction of a ring cut from a large coronary artery. De Bonis and Susanna also confirmed Langendorff's observation that adrenaline causes relaxation of such a ring, so that in this case the action of the two principles is again contrasted.

My own experiments were made with the isolated heart of the rabbit, perfused with oxygenated Locke-Ringer solution, by Langendorff's method as modified by Locke. There are several errors involved in the measurement of the coronary outflow from such a preparation. These have recently been discussed by Wiggers. The outflowing Ringer's fluid always accumulates to a certain extent in the right auricle and ventricle, and, as Schäfer pointed out, a certain amount may pass the semi-lunar valves and so reach the left ventricle. With small hearts I have not found that these defects seriously disturb the *average* rate of outflow: the principal drawback is that the dripping of the fluid from the heart is rendered irregular by the accumulation of fluid in the right side of the heart during diastole, and its ejection by the systole. With a small, rapidly-beating heart the quick and irregular succession of small drops which results can be averaged and converted into a regular series of large drops by a simple device. I used a large glass funnel, placed immediately beneath the recording lever. A skein of threads, hanging loosely from the heart and lever into the mouth of the funnel, ensured the delivery

into it of all the fluid leaving the heart, without at all interfering with the record of the contractions. The funnel was fixed in an inclined position and over the lower opening of the stem was drawn a short length of rubber tubing, the diameter of which could be reduced by a clip. This device converts an irregular series of drips and splashes into a regular series of large drops, which fall at a constant rate so long as the average rate of the drippings from the heart remains constant. These large drops were recorded on the smoked drum by the ordinary arrangement of receiving and recording tambours. When the beat of the heart and the rate of the coronary outflow, as shown by the drop recorder, had become constant, a small quantity of the filtered and warmed pituitary extract was introduced into the bulb of the heart-cannula by means of a hypodermic syringe, the needle being thrust through the wall of the rubber tube leading to the cannula. Fig. 1 shows a typical effect. It will be seen that the outflow from the coronary sinus becomes very much slower as soon as the extract reaches the heart. The effect shown in the figure is quite typical, and I know of no other drug which, in doses not immediately fatal to the heart-muscle itself, will produce so pronounced a constriction of the coronary arteries. That the effect is genuinely due to constriction, and not to viscosity or mechanical accident, can easily be ascertained from the fact that a second dose, introduced when the effect of the first has subsided, produces a very small change in the rate of outflow. This is quite in accordance with the observation, first made by Howell (25), that a second dose of the extract, given intravenously when the effect of a first large dose has passed off, produces hardly any rise of arterial blood-pressure.[1]

One other point needs mention. It is clear from what has been said above that a weakening or stoppage of systole might lead to an apparent temporary retardation of the coronary outflow by allowing accumulation in the right side of the heart. The phenomenon illustrated is not of that kind. It is a prolonged effect, which persists to some degree for upwards of half an hour after the injection, and its maximum coincides with a phase of increased ventricular activity. There is no room for doubt, therefore, that the coronary arterioles afford another example of an arterial area slightly, if at all affected by adrenaline, stimulated to intense constriction by pituitary extract.

The effect on the ventricular beat of the isolated heart

1. It is of interest to note that Dr. W. H. Harvey, to whom I communicated my observation of the constricting effect of pituitary extract on the coronary arteries, has produced sclerotic changes in these arteries by repeated injections of the extract.

Figure 1.—Ventricular beat and flow through coronary vessels of the isolated heart of a rabbit. Effect of adding 5 minims of pituitary extract to the perfusing Locke-Ringer solution.

Scale ½ linear.

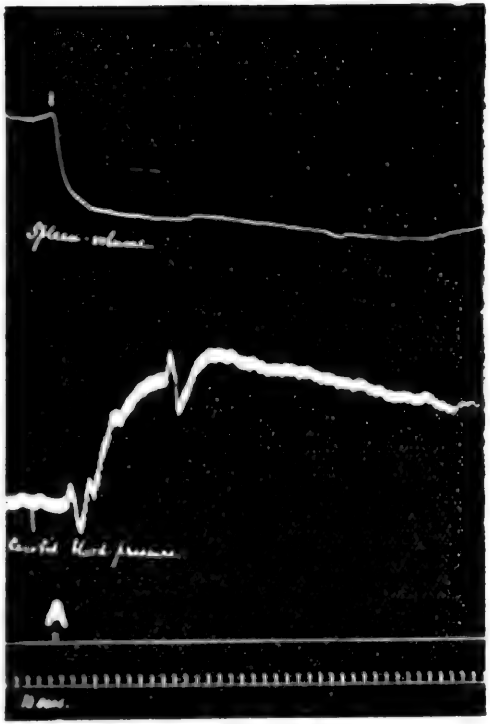
can also be studied in fig. 1. It will be seen that, immediately after the injection, it becomes slightly slower and considerably more vigorous: later, with persistent retardation, it becomes weaker than before the injection. Similar effects, in the same order, have been previously described by Hedbom (17) and by Cleghorn (18). It is difficult, however, to decide how far these changes in ventricular activity are due to primary action on the cardiac muscle, how far to reduction of the oxygen supply by coronary constriction. Neither effect is modified by previous atropinisation, so that there can be no question of the peripheral vagus-mechanism being concerned. There is further, in the case of the effect on the heart-beat, as in that of the coronary constriction, no resemblance whatever to the effect of accelerator nerves or of adrenaline. The safest conclusion is to regard the action on the coronary arteries as certainly a primary effect of the extract, that on the heart-beat as probably in part due to direct effect on the heart-muscle, and in part secondary to the altered rate of coronary perfusion. It should be noted, in this connection, that under conditions of natural circulation, in which the effect of coronary constriction would be antagonised by the great rise of systemic pressure, the secondary weakening of the beat is not usually observed.

The renal arteries. Schäfer with Magnus (19), and later with Herring (5), found that the kidney expanded when pituitary extract was injected intravenously. It was of interest, therefore, to examine the effect of pituitary extract on the rate of perfusion through the renal vessels. The perfusion was made with oxygenated Ringer's solution under constant pressure, as for the isolated heart, the outflow from the renal veins being measured by the drop-counter. The kidneys used were those of cats and dogs. Both kidneys of the cat were perfused, the cannulae being inserted into segments of aorta and vena cava. From the dog one kidney was used, with cannulae in the renal artery and vein. The pituitary extract was added by injection into the circulating Ringer's fluid. The following results were obtained:—

Injection of Pituitary Extract			Rate of Outflow in Drops per 20 seconds	
			Before injection	After injection
Experiment I.—Cat.		5 minims	34	20
Experiment II.—Cat.	1st.	5 minims	39	27
	2nd.	10 minims	29	31
Experiment III.—Dog.	1st.	5 minims	24	20
	2nd.	10 minims	20	22

It will be seen that the first injection causes in each case a decided though small constriction. The genuineness of the phenomenon is again shown by the failure of second injections, which even slightly reduce the resistance of the constricted arteries. Similar results were obtained by Houghton and Merrill (24), in the course of experiments made to determine whether the extract locally excites the renal epithelium to secretion. On the other hand Pal states that isolated rings of the proximal portion of the renal artery were constricted, while rings from more peripheral portions were relaxed by the extract. On the whole the evidence obtained with isolated organs suggests that the marked swelling of the kidney in its natural relations must be chiefly due to a relative insensitiveness of the renal arteries towards the vaso-constrictor effect of the extract. It might seem, at first sight, that even this implied, as Pal concludes, an action of the vaso-constrictor principle on some nervous structure, and not on the muscular coats of the arteries themselves. This, however, is by no means the only instance of an exceptional reaction of the renal arteries towards general stimulants of plain-muscle contraction. The various drugs of the digitalis series, for example, injected in small doses, cause expansion of the kidney and diuresis, especially in the rabbit; but the result of most experiments on the artificial perfusion of these drugs through the vessels of the excised kidney, especially of the dog and the cat, has been to demonstrate a marked constrictor action even on the vessels of that organ. There is no reason at all for supposing that these drugs act on nervous structures, and there is as little in the case of the pituitary extract. The anomalous reaction of the kidney vessels in their natural relations is clearly a similar phenomenon to their reaction to the digitalis series; but since the pituitary extract acts more powerfully on the arterioles and less on the heart than digitalis and its allies, the phenomenon is presented by the former in an exaggerated form.

The Spleen. The spleen may be regarded, in so far as its contractile activity is concerned, as belonging to the circulatory system. Schäfer and Magnus showed that pituitary extract caused contraction of the muscular capsule. I have repeated this observation with a like result. A plethyomographic record of the effect is shown in fig. 2.



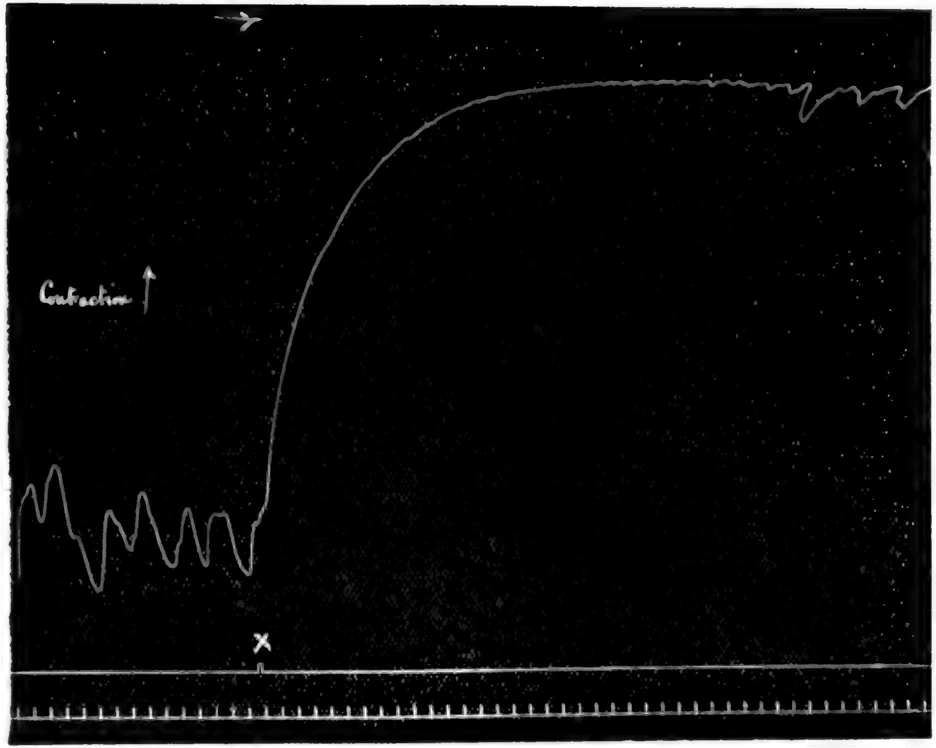


Figure 3. Contractions of isolated horn [illegible] cat [illegible] pregnant. At [illegible] 3 drops of [illegible] extract were added to the 200 c.c. of Ringer [illegible] in the bath. Time 10 seconds. Scale, ½ linear.

III. The Uterus

In a paper on another subject (22) I mentioned incidentally the powerful uterine contraction produced by pituitary extract. I have since extended the observation, finding, as expected, that the action, like that on the arteries, is possessed by extracts of the posterior lobe only.

Bell and Hick, working with the extract which I myself used, appear to have obtained a comparatively small effect on the rabbit's uterus in the resting (i.e., non-pregnant and non-oestrous) condition. This is quite contrary to my own experience. They worked exclusively with the rabbit. This animal is not really suitable, however, for our present enquiry, since its uterus responds, under all conditions, to the stimulus of sympathetic nerves or adrenaline, by contraction. In the cat, on the other hand, as was shown independently and almost simultaneously by Cushny (20), by Kehrer (21), and by myself (22), the uterine tone and contractions are inhibited in the non-pregnant, stimulated in the pregnant animal, by sympathetic nerves or supra-renal preparations. I regard it, then, as of great significance that in the uterus of the cat, as well as in that of the dog, the guinea-pig, the rat, and the rabbit, I have always observed, in all functional conditions, powerful tonic contraction as the effect of applying pituitary extract. The results were obtained by intravenous injection into the anaesthetised or brainless animal, and also by Kehrer's method of adding the extract to a bath of warm oxygenated Ringer's solution, in which the isolated horn of the uterus was so suspended as to pull on a recording lever. The effect, under these conditions of adding a few drops of pituitary extract to the 200 c.c. of Ringer's solution in the bath, is illustrated in figs. 3 and 4. So little, in my experience, is the effect dependent on the condition of the uterus as regards oestrum or pregnancy, that the uterus of a virgin, half-grown cat responded to the pituitary extract by as marked a tonic contraction as was given by any of the numerous pregnant or multiparous organs examined.

The effect of pituitary extract on the uterus, then, shows again the absence of parallelism to the effects of sympathetic nerves, the effect of the extract being always tonic contraction, even when stimulation of the hypogastric nerves produces pure inhibition of tone and rhythm.

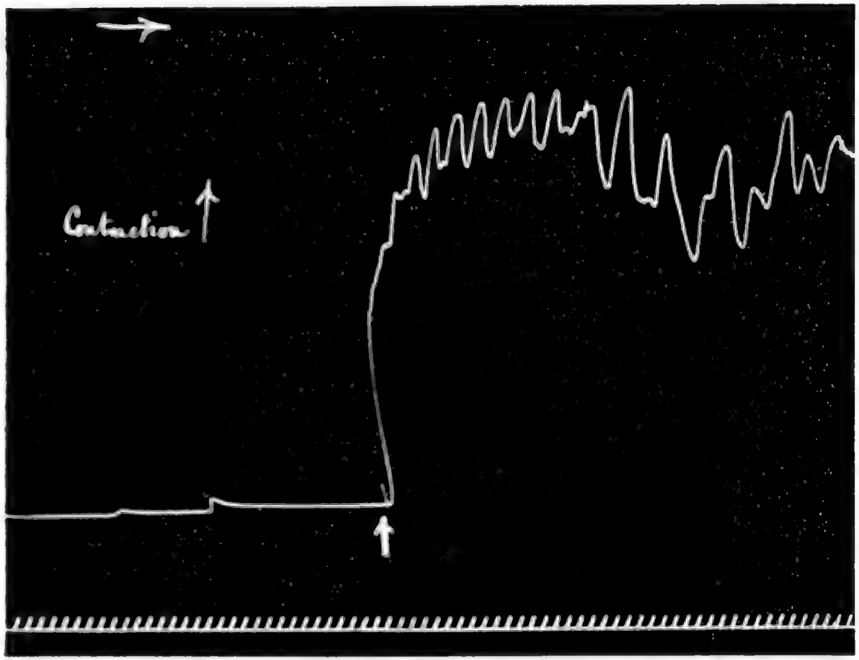


Figure 4.—A record, similar to that of Fig. 3, from the uterus of a pregnant guinea-pig. At ▲ 3 drops of pituitary extract were added to the bath. Scale, ½ linear.

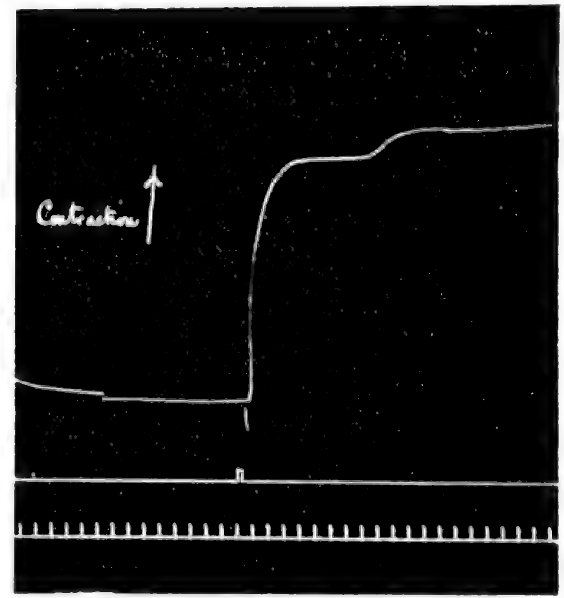


Figure 5.—Isolated retractor penis of the dog. Effect of adding 0·5 c.c. pituitary extract to the bath. Scale, ½ linear.

IV. Other Organs Containing Plain Muscle

The intestines and the urinary bladder give no such marked response to the pituitary extract as the organs hitherto mentioned. In a dog anaesthetised with A.C.E. mixture I observed, indeed, a distinct inhibition of intestinal movements when the extract was given intravenously, even when the splanchnic nerves were cut. This might be regarded as indicating a similarity of action to sympathetic nerves. An isolated loop of intestine, however, the rhythm and tone of which are immediately inhibited by adrenaline, contracts, though but feebly, when pituitary extract is added to the bath. It is probable, therefore, that the inhibition, seen under normal conditions of circulation, is due to the intense anaemia which the vaso-constrictor action of the extract produces.

The bladder of the cat, when the extract is injected intravenously, usually exhibits a temporary weakening, followed by more prolonged increase of tone. Neither is of any great extent. A guinea-pig's bladder, suspended in the Ringer-bath, contracted feebly when pituitary extract was added.

The plain muscular coats of the intestines and the bladder contract, then, like other plain muscle, in response to pituitary extract, but their sensitiveness thereto is small in comparison to that of some organs. The retractor penis of the dog, a convenient sheet of plain muscle for examination in the Ringer bath, contracts, as might be expected, when the extract is added (fig. 5).

No effect could be detected on pilo-motor muscles or on the mammalian pupil.

V. Gland Cells

Schäfer and Herring found that the extract caused secretion neither of saliva nor pancreatic juice, which observations I have confirmed. In its failure to evoke salivary secretion the extract is again contrasted to adrenaline. The profuse flow of urine which the extract causes, as first shown by Schäfer, in conjunction with Magnus (13) and with Herring (3), can hardly be regarded as a true glandular secretion.

VI. The Action after Ergotoxine

I have shown (23) that the specific ergot alkaloid ergotoxine, when injected intravenously in certain doses, annuls all motor effects of sympathetic nerves and adrenaline, so that the latter produces, in the cat,

a fall of blood-pressure and relaxation of the pregnant uterus in place of the customary rise and contraction. Ergotoxine may be given, however, in any quantity without affecting the contraction of arterial and uterine muscle produced by a subsequent injection of pituitary extract (fig. 6).

Action of Enzymes, etc., on the Extract

Schäfer and Herring (5) state that peptic digestion reduces the action of the extract on the blood-pressure without affecting the action on the kidney, but that neither action is affected by tryptic digestion. They also obtained results which they regarded as indicating that oxidation by H_2O_2 destroys the pressor action more quickly than the diuretic action. Certain obvious precautions seem to have been omitted: there is no indication that they controlled the activity of their enzymes or the response of their animal. A negative result should obviously not be accepted as indicating destruction of the agent unless a positive effect could subsequently be obtained with the untreated extract. Adopting these precautions I have failed to confirm them on all points. Digestion for twenty-four hours with a peptic extract of proved activity and 0·2 per cent. HCl failed to alter in any perceptible degree the pressor or diuretic action of my extract. I can only conclude that the peptic extract used by Schäfer and Herring contained some antagonistic depressor substance, or that their animal was for some reason unresponsive to the pressor effect. On the other hand every active preparation of trypsin which I have tried has reduced the action on the blood-pressure and on the urinary flow practically to *nil* after a few hours' digestion. Commercial trypsin, 'liquor pancreaticus,' pure pancreatic juice obtained by secretin and activated by enterokinase—all gave the same result. In all cases a subsequent injection of the original extract produced the usual rise of blood-pressure and acceleration of the flow of urine (figs. 7 and 8). It may be suggested, in the absence of evidence for control on that point, that Schäfer and Herring were using an inactive preparation of trypsin: at least it is clear that the tryptic preparations used by me contained something which was not present in theirs. In my experience oxidation with H_2O_2 failed likewise to discriminate between the pressor and diuretic activities. Both effects were smaller after oxidation than those produced by a subsequent injection of the original extract; but that either had suffered greater change than the other was not apparent.

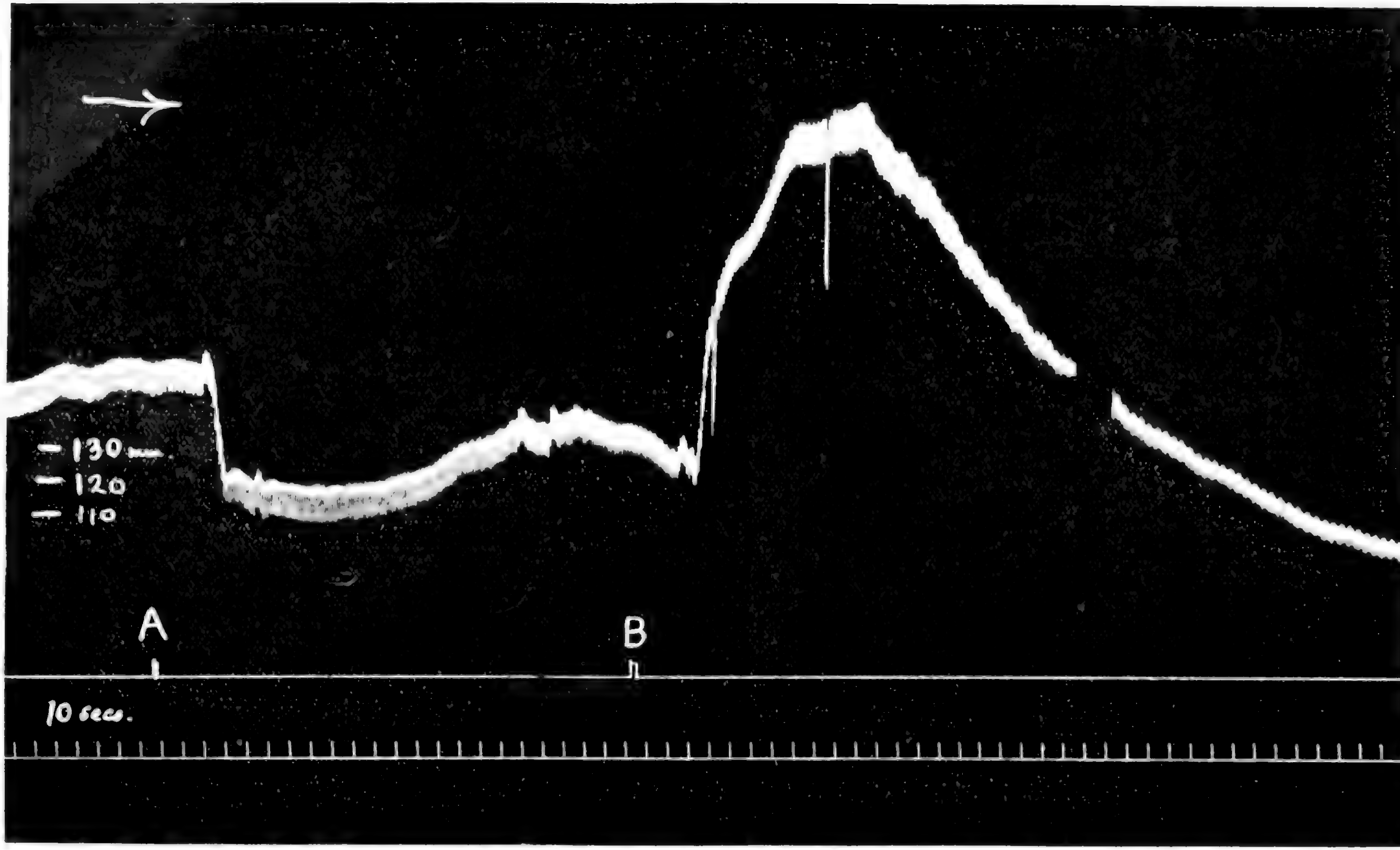
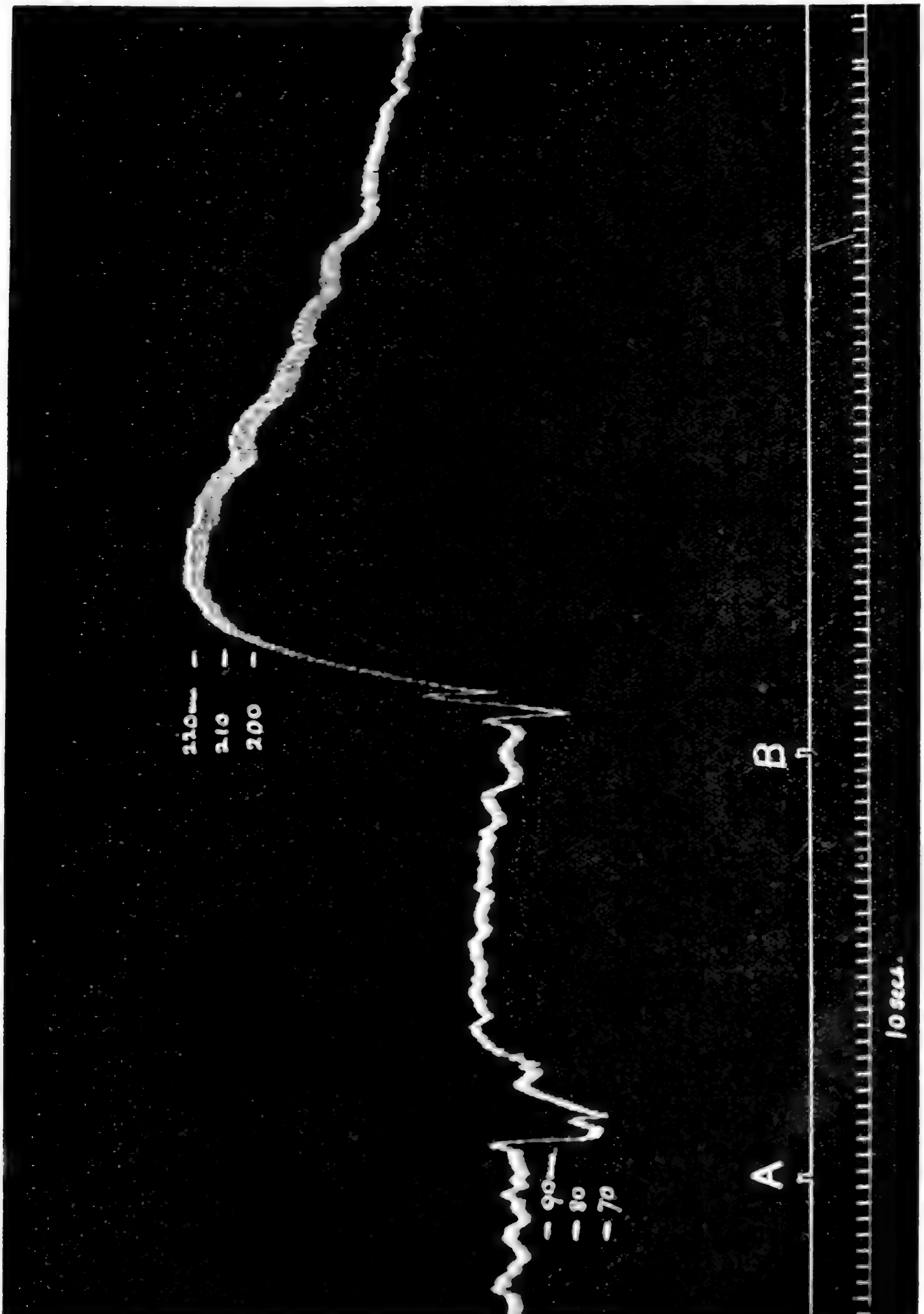


Figure 6. Carotid blood-pressure of pithed cat. 5 mgms. ergotoxine phosphate injected previously. Injections:
At A—0·1 mgm. adrenaline.
At B—2 c.c. pituitary extract.
Scale, ½ linear.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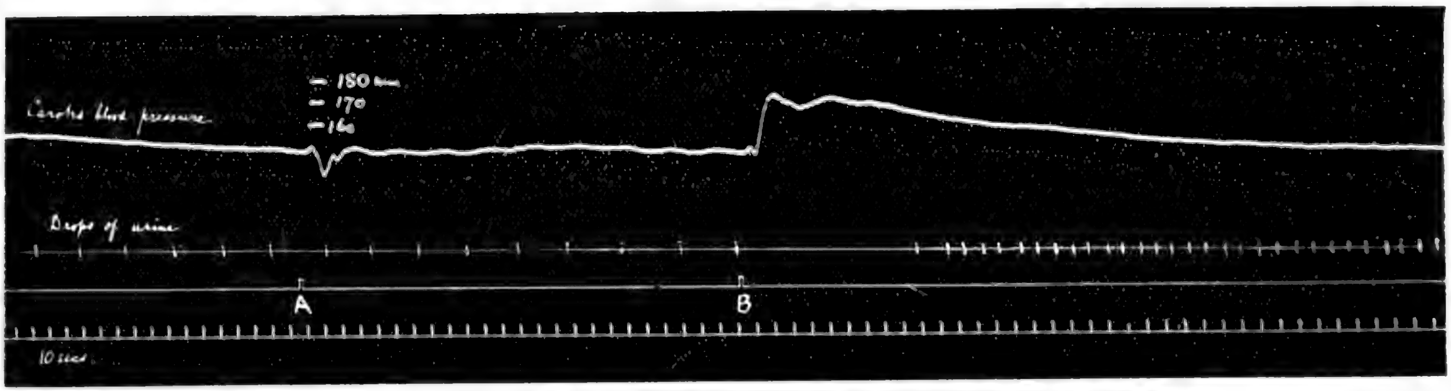

Figure 8.—Carotid blood-pressure and drop-record of urine (bladder cannula) of cat (ether). Injections as in Fig. 7:

At A—1 c.c. extract digested with trypsin.
At B—1 c.c. extract incubated with boiled trypsin.

Scale, ½ linear.

Excretion. Attempt to Produce Immunity

The fact, discovered by Howell, that second doses are relatively ineffective, suggests that the active principle is not readily destroyed or rendered inactive in the body. I found that the urine of a cat, excreted in response to an injection of the extract, had a pressor action, like a dilution of the extract, when tested on another cat (fig. 9). Probably the active principle, therefore, is at least to some extent excreted unchanged.

The refractory state to further injections has nothing to do with a true 'immune' reaction. In the serum of a rabbit, treated for a month with increasing injections of the extract, I could distinguish no trace of a body neutralising the physiological activities of the extract.

Discussion of the Results

It is clear from the foregoing that the characteristic action of extracts of the posterior lobe of the pituitary body is stimulation of plain muscle fibres. Different organs containing plain muscle show a varying sensitiveness of response to the extract, the arteries, the uterus and the spleen being conspicuously affected. This unequal distribution of effect cannot, however, in any way be related to inequalities of innervation by nerves of the true sympathetic or of the autonomic system as a whole. Ergotoxine, which excludes motor effects of true sympathetic nerves, and of drugs acting through those nerves or like them, leaves the action of pituitary extract intact. Neither atropine nor curare affects its direct action in any degree. The muscle of the mammalian heart is possibly affected to some extent by the extract, apart from effects secondary to constriction of the coronary arterioles: Herring's observations on the frog's heart render this most probable. No effect could be detected on the response of voluntary muscles, either to direct or indirect stimulation. The active principle is then essentially a stimulant of involuntary, and especially of plain muscle.

The question of the diuretic effect needs some further discussion. Houghton and Merrill (24) have recently taken the somewhat extreme view that this is entirely secondary to the rise of blood-pressure. They state that the rise of blood-pressure produced by adrenaline is accompanied by a similar diuresis. This latter observation is directly opposed to the experience of others, and I have never myself been able to confirm it. Further it was shown quite clearly by Schäfer and Herring that a second injection of pituitary extract may cause distinct

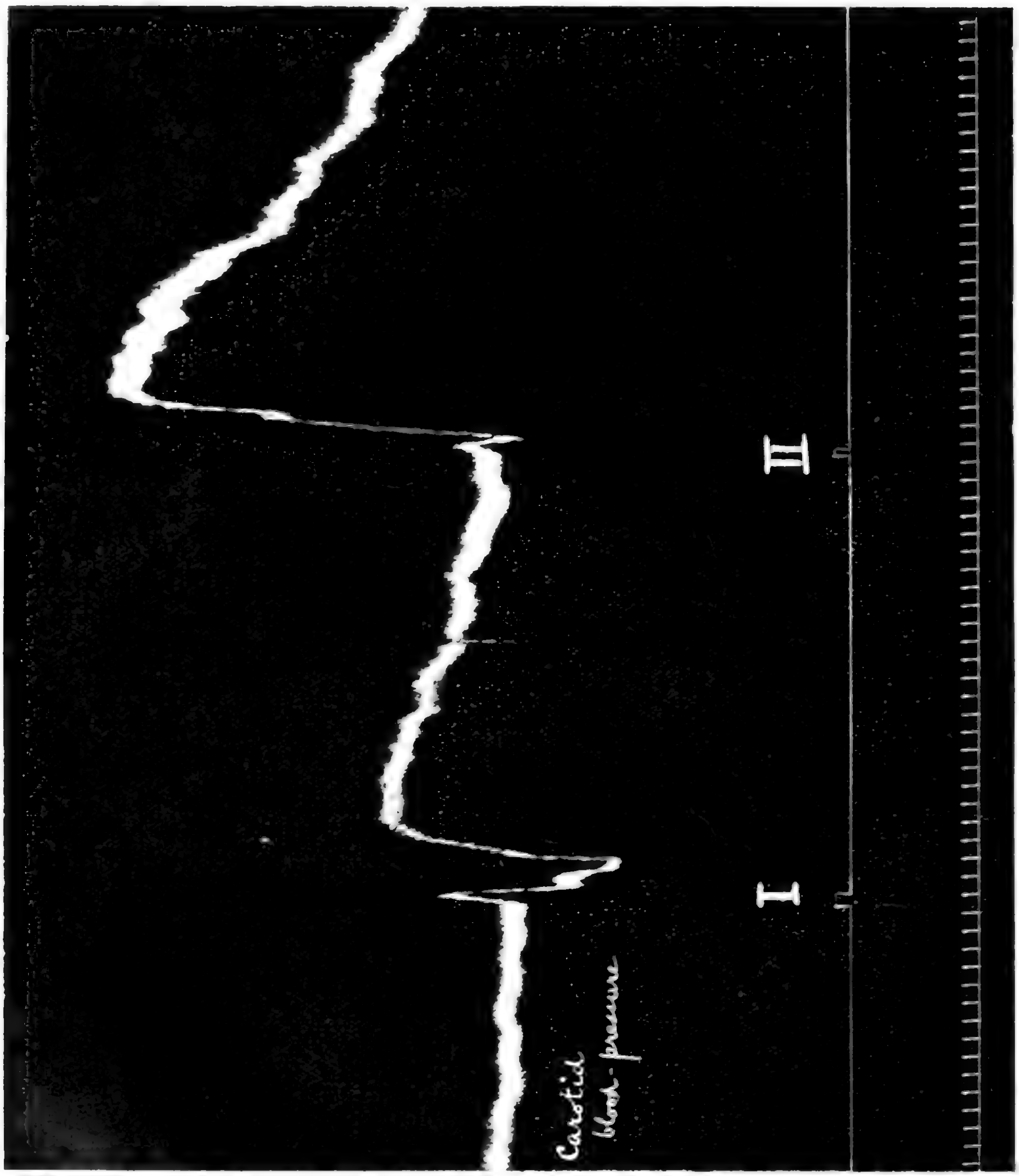

Figure 9.—Carotid blood-pressure of pithed cat. Intravenous injections:
At I.—8 c.c. of normal cat's urine.
At II.—8 c.c. of urine collected after injection of 4 c.c. pituitary extract. (50 c.c. in all collected during 2 hours).

diuresis without any perceptible rise of blood-pressure. While such an observation, which I have been able repeatedly to confirm, sufficiently disproves the statement that the diuresis is secondary to and runs parallel to the actual rise of systemic pressure, it does not remove the possibility of the dependence of the diuresis on vascular effects. A redistribution of the blood in the system, caused by the comparative irresponsiveness of the renal arterioles, is conceivable without actual rise of general systemic pressure, especially if the arterial constriction is accompanied by weakening of the heart's action, due to the depressor constituent which the extract always contains, the action of which, moreover, is much more evident in the case of a second injection.

The differential action of enzymes and oxidation on the supposed pressor and diuretic principles, alleged by Schäfer and Herring, has not been confirmed in my experiments. On the contrary I have found that whatever destroyed one action destroyed both. Their other evidence for the existence of two principles seems to me also inadequate. They lay stress on the difference in the time relations between the two effects and the relatively greater effect of second injections on diuresis. The difference in time-relations of a diuretic and pressor effect is, however, a familiar phenomenon in cases where there can be no question of the presence of more than one active principle. If strophanthin, for example, be injected intravenously into a dog or cat, the immediate effect on the diuresis is usually a distinct retardation: later, as the rise of arterial blood-pressure passes off, there is generally a secondary acceleration which often persists after the blood-pressure has regained its original level. A similar sequence of events was recently observed by P. P. Laidlaw and myself in experiments, in course of publication, on the action of a pure, crystalline active principle from Apocynum. Such a difference in time-relations cannot, therefore, be accepted as necessitating the presence of two principles. The relatively greater efficacy of a second injection in causing diuresis as compared with its pressor effect can also be interpreted in another way, as indicated above. The blood-pressure tracing is complicated by the presence of the heart-depressing principle: it is not a fair index of the degree of vaso-constriction in this instance. An apparently greater relative efficacy of second injections can also be observed in the case of the uterus, when the effect on that organ is compared with that on the arterial pressure. I have frequently seen, as the result of a second injection, marked contraction of the uterus accompanying a very slight or no rise of blood-pressure.

It does not seem justifiable, however, to draw from this observation the conclusion that the principle acting on the plain muscle of the uterus is different from that which acts on the plain muscle of the arteries. It is, of course, true that nothing short of the isolation of a single pure principle, producing both pressor and diuretic effects, would make the view that two principles exist untenable. While awaiting further evidence, however, the conception of both effects as due to one principle seems to me adequate and simpler.

Conclusions

1. The action of extracts of the posterior lobe of the pituitary body is a direct stimulation of involuntary muscle, without any relation to innervation. The action is most nearly allied to that of the digitalis series, but the effect on the heart is in this case slight, that on plain muscle intense.

2. The active principle is excreted in the urine.

3. No true immune reaction is produced by repeated injections of the extract.

4. The evidence advanced in proof of the existence of separate pressor and diuretic principles is inadequate.

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A METHOD FOR THE ESTIMATION OF THE UREA, ALLANTOIN, AND AMINO ACIDS IN THE URINE

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In investigations involving the determination of the distribution of nitrogen in the urine, the separation of the various nitrogenous constituents is a matter of no little difficulty, and involves so much time that it is almost impossible to carry through any prolonged series of observations.

The object of the present investigation is to determine how far the distribution of nitrogen can be more rapidly determined indirectly by taking advantage of the differences in solubility and stability to reagents of the various substances.

The chief substances in which nitrogen occurs in the urine are:—Urea, purin bodies including uric acid, creatinin (with or without creatin), ammonia, allantoin, amino acids (including hippuric acid), sulphur-containing bodies and bodies of unknown composition.

The present investigation is confined to the determination of urea, allantoin and amino acid nitrogen.

For the determination of *Urea* Bohland (1) recommended the precipitation of the other nitrogen-containing substances by phosphotungstic and hydrochloric acids. The urea was then estimated in the filtrate. He claimed that the results got by this method gave only urea nitrogen and ammonia nitrogen, all other nitrogen-containing bodies being precipitated.

Later Schöndorff (2) showed that the amino acids (glycocoll, leucin, &c.) are not precipitated by phosphotungstic and hydrochloric acids. He also showed that while creatinin is precipitated, as Bohland had already found, creatin is not. Allantoin also is not precipitated; a statement later confirmed by Mörner (3).

Pfaundler (4) experimented with various samples of phosphotungstic acid and found that, if Merck's preparation is used, ammonia is precipitated.

The nitrogen obtained by this method therefore includes urea, amino acids (with hippuric acid), creatin and allantoin nitrogen.

Mörner and Sjöqvist (5) recommended a method for the estimation of urea in which the urine is precipitated with a saturated solution of barium chloride in which five per cent. of barium hydrate is dissolved, and an alcohol ether mixture containing two parts alcohol to one of ether. After twenty-four hours it is filtered, the filtrate evaporated at 50° to small bulk, after the addition of a pinch of magnesium oxide to drive off the ammonia, and the nitrogen in it estimated by Kjeldahl's method. Creatinin, hippuric acid, and also some amino acids (leucin, bile acids) are not precipitated, and their nitrogen is thus included in the amount obtained by this method.

Folin (6) proposed a method of heating the urine with crystallised magnesium chloride and hydrochloric acid. At the temperature thus employed—150° C.—urea and allantoin alone are decomposed to give off ammonia. Thus the nitrogen obtained by this method includes allantoin nitrogen in addition to urea nitrogen and ammonia nitrogen.

Mörner, in a more recent paper (7), develops what may be called the Mörner-Folin method by which urea alone yields its nitrogen. The bulk of the nitrogen-containing substances are precipitated as described above, with barium chloride, barium hydrate and alcohol ether solution, but the evaporated filtrate is heated with magnesium chloride and hydrochloric acid as in Folin's method. Allantoin is by this procedure almost entirely removed before heating with magnesium chloride, thus avoiding Folin's error. The creatinin, &c., which are not precipitated in the Mörner method are not decomposed in the Folin method. Thus the Mörner-Folin method gives only the nitrogen which is contained as urea.

In the present investigation a slight modification of this method was adopted, a modification first employed by Underhill and Kleiner (8), viz., the use of an alcohol ether solution containing equal parts of alcohol and ether, since as Haskins (9) had previously observed, allantoin is more insoluble in such a solution than in the one employed by Mörner. In addition a few drops of hydrochloric acid were at once added before the evaporation of the alcohol ether filtrate, thus preventing the escape of ammonia, the nitrogen in which is thus included in the results. This addition of acid was also recommended by Folin, instead of driving off the ammonia by means of magnesium oxide as practised by Mörner.

The method in detail as employed by me is, 5 c.c. of the urine are mixed with 5 c.c. barium chloride, barium hydrate solution, and 100 c.c. alcohol-ether (50 c.c. absolute alcohol, 50 c.c. ether). After twenty-four hours it is filtered, an aliquot portion of the filtrate is taken, and a few drops of hydrochloric acid immediately added. This is then evaporated almost to dryness at a temperature of 50° C. in an apparatus described by Haskin; 20 grs. magnesium chloride are then added and a small piece of paraffin wax to prevent frothing, and the whole is heated for three hours on an electric heater described by Cathcart (10).

The direct determination of *Allantoin* has always proved difficult and unsatisfactory. The similarity between its properties and those of urea renders complete separation extremely difficult. Poduschka's method (11), in which allantoin is precipitated by silver nitrate in alkaline solution, is comparatively simple, though somewhat lengthy, but, as Salkowski says, the precipitation of the silver compound with ammonia is one of the most difficult of chemical procedures.

Wiechowski (12) proposed using mercuric acetate, which precipitates allantoin but not urea, instead of the usual mercuric nitrate, by which urea too is precipitated. Allantoin when thus precipitated shows a strong tendency to crystallise. The details of the method are, however, long and troublesome, though giving accurate results, and it is impracticable for a long series of daily observations.

For the estimation of the *Amino acids* various methods have been suggested.

Fischer and Bergell (13) proposed a direct method which depends on the power of napthol-sulphonic acid to combine with amino acids. The urine was shaken with an ethereal solution of β napthalene sulpho chloride and the amino acid compound subsequently precipitated with hydrochloric acid. This method was later modified by other workers. Glaessner (14), in criticising the method, states that he, as others also, did not recover on an average more than 60 per cent. of the amino acids.

Neuberg and Manasse's direct method (15) depends on the fact that on shaking a urine with a strongly alkaline solution of α-napthol isocyanate, hydantoic acid is formed, which is precipitated after acidification. Glaessner found this method not at all constant. Some good results were obtained, but in general they were quite unreliable.

Pfaundler (16) endeavoured to devise an indirect method for the estimation of amino acids. His method was to precipitate the urine first with phosphotungstic acid. The precipitate and filtrate were then each

heated with phosphoric acid. The urine nitrogen was thus divided into four fractions.

I. Substances precipitated by phosphotungstic acid.
 (*a*) Nitrogen easily removed by heating with phosphoric acid.
 (*b*) Nitrogen not thus easily liberated.

II. Substances not precipitated by phosphotungstic acid.
 (*a*) Nitrogen easily removed by heating with phosphoric acid.
 (*b*) Nitrogen not thus easily liberated.

Of these four fractions, II (*a*) consists mainly, if not entirely, of amino acids.

Kruger and Schmid's method (17), also an indirect one, was based on the fact that amino acids do not give off ammonia when heated with concentrated sulphuric acid at 160° to 180° C. They estimated the nitrogen in the filtrate from phosphotungstic acid and also the nitrogen which was obtained after heating with half the volume of concentrated sulphuric acid. The difference between these two should correspond to the amino acid nitrogen.

Glaessner (18) proposed a somewhat similar method for the estimation of amino acids. He precipitated with phosphotungstic acid and evaporated the filtrate to dryness at a low temperature. The residue was freed from water and then extracted with alcohol-amyl alcohol for six hours. It was then filtered and a Kjeldahl nitrogen estimation done on the residue. This gave the amino acid nitrogen. The difficulty of this method is to ensure that the urea is completely dissolved.

Present Methods

It appeared possible that the combination of certain of these methods —Bohland, Folin, Mörner-Folin—might yield definite results as to the distribution of nitrogen, and the method which I employ is as follows:—

Estimations of the nitrogen present are made by these three methods, and by the differences between them the nitrogen in urea, allantoin and amino acids is determined.

(*a*) Bohland nitrogen, using Merck's phosphotungstic acid, which leaves unprecipitated amino acids, hippuric acid, creatin, allantoin and urea.

(*b*) Folin nitrogen, which includes urea, allantoin and ammonia nitrogen.

(*c*) Mörner-Folin nitrogen, which includes urea and ammonia nitrogen.

The ammonia nitrogen was separately estimated by Folin's method and subtracted from (*b*) and (*c*).

The difference then between the nitrogen of (*a*) and (*b*) should give the amount of amino acid nitrogen and creatin nitrogen present, hippuric acid being included as an amino acid. The creatin can be determined separately by Folin's method.

The difference between the nitrogen of (*b*) and (*c*) should give the amount of allantoin nitrogen present.

To verify the method estimations were made on solutions containing varying amounts of urea, allantoin and amino acids. The nitrogen in each solution was determined by Kjeldahl's method.

I. A solution was made consisting of 25 c.c. of a 2 per cent. solution of urea, 25 c.c. of a 0·5 per cent. solution of allantoin, and 10 c.c. of a 0·5 per cent. solution of alanin. 5 c.c. of this solution contained 0·0016 grs. nitrogen as allantoin and 0·0022 grs. nitrogen as alanin.

Bohland	nitrogen required	9 c.c.	$\frac{N}{10}$ acid	=	0·0126 grs. nitrogen	
Folin	,,	7·4 c.c.	,,	=	0·0104	,,
Mörner-Folin	,,	5·05 c.c.	,,	=	0·0088	,,

B. – F. = 0·0022 grs. nitrogen = 100 p.c. alanin nitrogen

F. – M.-F. = 0·0016 ,, = 100 p.c. allantoin ,,

II. Solution consisted of 25 c.c. of a 0·5 per cent. solution of allantoin, 25 c.c. of a 1 per cent. solution of glycocoll, 25 c.c. of a 2 per cent. solution of urea, and contained 0·042 grs. nitrogen as allantoin and 0·044 grs. nitrogen as glycocoll.

Folin	nitrogen required	13·1 c.c.	$\frac{N}{10}$ acid	=	0·275 grs. nitrogen	
Mörner-Folin	,,	8·9 c.c.	,,	=	0·234	,,

F. – M.-F. = 0·041 grs. nitrogen = 97·6 p.c. allantoin nitrogen

III. Solution consisted of a mixture of a 2 per cent. solution of urea and a 0·5 per cent. solution of allantoin, 5 c.c. of which contained 0·0034 grs. nitrogen as allantoin.

Folin	nitrogen required	15·25 c.c.	$\frac{N}{10}$ acid	=	0·02135 grs. nitrogen	
Mörner-Folin ,,	,,	12·9 c.c.	,, ,,	=	0·0181	,,

F. – M.-F. = 0·00325 grs. nitrogen = 95·5 p.c. allantoin nitrogen

IV. Solution consisted of a mixture of a 2 per cent. solution of urea and a 1 per cent. solution of glycocoll, 5 c.c. of which contained 0·0035 grs. nitrogen as glycocoll.

Bohland nitrogen = 15·2 c.c. $\frac{N}{10}$ acid = 0·0212 grs. nitrogen

Folin ,, = 12·7 c.c. ,, = 0·0178 ,,

B. – F. = 0·0034 grs. nitrogen = 97 p.c. glycocoll nitrogen

V. Solution consisted of a mixture of a 0·25 per cent. solution of alanin and a 1 per cent. solution of urea, containing 0·45 grs. nitrogen as urea, and 0·037 grs. nitrogen as alanin.

Bohland nitrogen = 34·15 c.c. $\frac{N}{10}$ acid = 0·478 grs. nitrogen

Folin ,, = 31·6 c.c. ,, = 0·442 ,,

B. – F. = 0·036 grs. nitrogen = 97 p.c. alanin nitrogen

These results are summarised in Tables I and II.

TABLE I

No. of solution	Grs. allantoin nitrogen	F. – M.-F.	P.c. allantoin	Solution contained
I.	0·0016	0·0016	100	Urea, alanin, allantoin
II.	0·042	0·041	97·6	Urea, allantoin, glycocoll
III.	0·0034	0·00325	95·5	Urea, allantoin

No. of solution	Grs. amino acid nitrogen	B. – F.	P.c. amino acid	Solution contained
I.	0·0022	0·0022	100	Urea, allantoin, alanin
IV.	0·0035	0·0034	97	Urea, glycocoll
V.	0·037	0·036	97	Urea, alanin

In order to ascertain whether these methods held when applied to urines, estimations were made on urines to portions of which known amounts of allantoin and amino acids had been added.

I. Setter's urine.

Folin nitrogen = 0·116 grs. nitrogen
Mörner-Folin ,, = 0·116 ,, ,,
F. – M.-F. = 0 ,, ,,
An allantoin solution containing 0·016 grs. nitrogen was added
Folin nitrogen = 0·1204 grs. nitrogen
Mörner-Folin ,, = 0·1036 ,, ,,
F. – M.-F. = 0·0168
Increase of difference = 0·0168 grs. nitrogen = 105 p.c. allantoin nitrogen

II. Goose's urine.

Folin nitrogen = 0·00266 grs. nitrogen
Mörner-Folin ,, = 0·0021 ,, ,,
An allantoin solution containing 0·0096 grs. nitrogen was added.
Folin nitrogen = 0·0116 grs. nitrogen
Mörner-Folin ,, = 0·0021 ,, ,,
F. – M.-F. = 0·0095 = 98·9 p.c. allantoin nitrogen

III. Goose's urine.

10 c.c. urine + 10 c.c. water.

Folin nitrogen = 0·00532 grs. nitrogen

10 c.c. urine + 5 c.c. water + 5 c.c. allantoin solution containing 0·0096 grs. nitrogen

Folin nitrogen = 0·01456 grs. nitrogen

Difference = 0·00924 grs. nitrogen = 95·6 p.c. allantoin nitrogen.

IV. Goose's urine.

15 c.c. urine + 15 c.c. water.
Bohland nitrogen = 0·0042 grs. nitrogen
Folin ,, = 0·00798 ,, ,,
B. − F. = 0·00378 grs. nitrogen
15 c.c. urine + 10 c.c. water + 5 c.c. glycocoll solution containing 0·0091 grs. nitrogen
Bohland nitrogen = 0·01344 grs. nitrogen
Folin ,, = 0·00798 ,, ,,
B. − F. = 0·00546 grs nitrogen
Increase of difference = 0·00924 grs. nitrogen = 101·5 p.c. glycocoll nitrogen

The results are summarised in Table III.

TABLE III.

No.	Grs. nitrogen added to urine	Grs. nitrogen determined	P.c. nitrogen	Remarks
I.	0·016	0·0168	105	Urine + allantoin
II.	0·0096	0·0095	98·9	Urine + allantoin
III.	0·0096	0·00924	95·6	Urine + allantoin
IV.	0·0091	0·00924	101·5	Urine + glycocoll

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ON THE NATURE OF THE SO-CALLED FAT OF TISSUES AND ORGANS

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The question of the nature of the fatty substances present in animal organs and tissues has of late years received considerable attention, and research in this direction has elicited some noteworthy facts. On the other hand, the general problem of fat metabolism in almost all its details still awaits solution, and though the processes undergone during fat absorption are now fairly well understood, we have absolutely no knowledge of the methods utilised in the body in connection with the katabolism of these substances. The recent interesting observations of Leathes and of Hartley point to the liver being an active agent in the preparation of fat prior to its final oxidation in the tissue cells; it is, however, not improbable that other organs may also be capable of participating in this preliminary desaturation of the fatty acid radicles.

NATURE OF SO-CALLED TISSUE FAT

For many years it has been recognised that fatty substances may be present in, or at least derived from, an organ which, on ordinary macroscopical or microscopical examination, appears to be absolutely fat-free, and gives no trace of reaction to the specific fat stains. This 'masked' fat can, however, be rendered visible under particular circumstances, and the question of its origin has given rise to one of the greatest controversies in the annals of pathological chemistry; it is now generally accepted as arising from a combination of fat and protein normally present in the tissue, the fat becoming evident only as the result of certain post- or ante-mortem changes, by which the compound is broken up and the fat liberated.

The recognition of the presence of this combined fat explained the fact that ordinary solvents, such as ether, are incapable of extracting all the fat from an organ, and it was found that better yields were obtained by the addition of auxiliary substances, such as alcohol and chloroform.

Various modifications have been suggested, but in most cases the general principle of fat extraction adopted and recommended by the different investigators depends on thorough extraction of the tissue by combinations of the above solvents with the aid of heat. That an organ can be freed from fat-like substances by the above means may be granted, but it is exceedingly doubtful whether the actual substances present in the extract represent with any degree of exactitude the fatty substances as they actually existed in the tissue prior to extraction. Of late, attention has been chiefly focussed on the fatty acids obtained, but the nature of the compounds in which these fatty acids are actually present in the tissues has been to a great extent lost sight of. It seems obvious, however, that a correct knowledge of the nature and disposition of the 'fat' in the animal organs is the only way by which a key to the difficult problem of fat metabolism is likely to be found.

Experiment shows that in many cases the greater part of the fat obtained is really not fat in the ordinary sense of the word, but to a great extent complex combinations of fatty acids with glycero-phosphoric acid and a nitrogen-containing compound—the so-called phosphatides.

Though it has been long known that tissues and organs contain 'lecithin,' it is hardly recognised that very much of the 'fat' they do contain may be present in this or in an allied form. What ordinary neutral fat can be extracted from an organ seems to be generally interstitial, and is present as stored fat, just as glycogen represents stored carbohydrate. On the other hand, fat which is actually being made use of by the living cells seems to be represented to a very great extent—if, indeed, not altogether—by phosphatides.

These substances, as pointed out by Heffter,[1] are exceedingly labile, and undergo partial decomposition when heated to a temperature of over 50° C.; even an acid reaction has a similar tendency. Under these circumstances it might be expected that the nature of the material obtained by the different extraction methods would vary in its composition, and investigation proves that this is actually the case. This fact in itself indicates that the general extraction methods do not suffice for the determination of what appears, after all, to be one of the fundamentally important points in such investigations—the general nature of the fatty substances originally present in the tissue. It is obvious that such methods as that recommended by Dormeyer,[2] in which

1. *Arch. f. exp. Pathologie,* XXVIII, p. 97, 1891.
2. *Arch. f. d. ges. Physiol.,* Vol. LXV, p. 90, 1907.

digestion with pepsin and hydrochloric acid is utilised to separate off the masked fat, must also result in more or less marked disintegration of the more labile fatty substances. The varying saponification values obtained in the following experiments show that there must be marked differences in the different extracts prepared by various methods.

Experiments with different Extraction Methods

Dog's liver was taken, and an equal amount used for each experiment. The final extracts obtained were then saponified in the usual way with alcoholic potash, and the saponification values compared.

(1) *Noel Paton's Method.*—Liver was cut up into small pieces and placed in methylated spirit for a week; the spirit was then poured off into an evaporating basin and the liver pounded in a mortar and thrown again into the spirit. The whole was then dried on a water bath, the temperature being always kept below 80° C. The contents of the basin were then put into a Soxhlets apparatus and extracted with ether for about 12 hours. The syrupy extract obtained was then dried and saponified.

Saponification value = 215.

(2) Liver was cut up and extracted for a week with cold alcohol; it was then treated with hot alcohol for several hours, and with ether as described on page 458. Ether extract was separated and the ether distilled off at a low temperature.

Saponification value = 248·2.

(3) Liver was thoroughly ground up with calcium sulphate until a fine dry powder was obtained. This was repeatedly extracted with ether in the cold. Ether was then distilled off at a temperature below 50°.

Saponification value = 234·84.

The essential features of these experiments, given in tabular form, are as follows, and show that both the nature of the extracting substances and the order in which they are used, play a part in determining the nature of the final extract obtained. While the saponification values are given as one instance indicating a difference in the extracts, there are other variations with regard to the amounts of free fatty acids, etc., present:—

Organ used	Nature of extraction	Saponification value of extract
Dog's liver in all experiments	Placed in methylated spirit for 7 days; dried at 80°. Total mass extracted with ether for 12 hours.	215
	First cold alcohol; then hot alcohol; then ether.	248·2
	Dried with $CaSO_4$. Extracted with cold ether	234·84

An extension of such experiments showed that not only are different results obtained by different methods of extraction, but that the same method may give variations in the nature of the substances obtained: the chief explanation seems to be the one that naturally presents itself, viz., that during the ordinary process of extraction, in which heat is invariably employed, there is, together with the abstraction of the fat-like substances from the tissues, a more or less well-marked disintegration of the extracted products themselves; the extent to which this destructive change becomes manifest depends no doubt to a great extent on the amount of heat utilised during the process.

Again, the above methods are absolutely useless for determining another most important point—the relative amounts of *free* and *combined* fatty substances in the tissues. It may well be that the extremely labile nature of these substances causes their extraction to be inevitably associated with a certain amount of change in their constitution, but it is obvious that this tendency is exaggerated by the use of hot solvents, and the only rational method would appear to be extraction at as low a temperature as possible. By a careful combination of solvents used in the cold, a fair idea can be obtained of the nature and disposition of these fatty substances in the tissues, and it is hoped to publish shortly a set of experiments giving information with regard to these important details.

In all our experiments it was noticed that the saponification values obtained were very high, and the following samples indicate the nature of the figures found in the case of different organs from the dog.

The animals were anaesthetised with chloroform, quickly bled, and transfused with saline. The organs were then removed, transfused separately, and then minced and weighed in the moist condition.

Method of Extraction

Tissues were extracted for several days with cold alcohol, the alcohol being changed several times. The residue was then treated for one to two hours with boiling alcohol, a reflux condenser being used. After removal of the hot alcohol, the residue was extracted several times with ether. Both alcoholic extracts were mixed, the alcohol evaporated, and the residue taken up in ether. This was mixed with the ether extract of the tissue, and after the chief part of the ether was driven off, residue was dried in vacuo over H_2SO_4. Saponification was carried out with alcoholic potash.

Saponification figures of 'fat' from organs of dog

	Dog A		Dog B
Blood	153·89	...	—
Connective tissue	223·9	...	195·2
Liver	231·6	...	230·5
Kidney	264·6	...	—
Heart	256·2	...	278
Muscle	254·5	...	264·5

These figures at once indicate that this extract cannot to any extent be composed of ordinary neutral fat, for the average figure for fats lies between 190 and 200; undoubtedly such substances as cholesterol are present to a varying extent, but these and other similar substances would tend to *lower* the saponification values. The occurrence of fatty acids of low molecular weight was considered, but no evidence of their presence could be obtained. The alternative explanation seemed to point to the presence of phosphatides as the cause of the high values, and investigation showed that this was the case. It occurred to B. Moore some years ago, when doing some work on liver fats, that the greater part of the 'fat' of this organ was often present in some form other than neutral fat or free fatty acids, and observations made by us at his request indicates that the same holds good for certain other organs; it is very probable that the free fatty acids found in extracts obtained by the ordinary methods are to a considerable extent disintegration products of tissue phosphatides. The high saponification values were found to be caused by combination of part of the sodium with phosphoric acid and glycero-phosphoric acid liberated during the process of saponification.

Every organ and tissue naturally contains more or less neutral fat in the interstices of its substance; but though it would seem that the preponderating portion of the 'fat' combined with protein in the bioplasm as masked fat is present as phosphatide, in addition some of the phosphatide is present in free form, perhaps as a phase in its passage to combination; free phosphatides would, on this view, constitute preliminary substances which subsequently pass on to actual combination in the tissues. In a normal organ, therefore, the less microscopical evidence there is of fat, the less neutral fat is actually present; while the combined fat—a phosphatide seems to represent one of those steps in that synthetical elaboration of fats which appears to be a necessary prelude to actual assimilation. It is not improbable that phosphatides represent a necessary step in the elaboration of fatty substances destined ultimately to undergo actual assimilation into living matter. That such substances

are essential for the vital processes seems indicated by their presence in all living cells hitherto investigated; it cannot be doubted that one of the steps which ordinary fat undergoes in the cell is a transformation into phosphatide, and probably in these bodies the desaturation of the fatty acid radicle is brought about. Whether this elaboration is necessary for the ultimate oxidation of all fats, or whether we have here wholly or in part a process somewhat analagous to the endogenous metabolism of protein, can as yet be but conjecture. The fact, however, that phosphatides contain practically all the constituents (even iron, according to Glikin) of nucleo-proteins, is not without significance, and it is not unlikely, as partly suggested by Hammersten, that they may be the source of the cell nucleo-proteins.

The marked amount of phosphatide obtained in two experiments in which pig's liver was used is shown by the following figures. The phosphatides were separated by means of acetone. As full details of similar experiments will be published later on, the general outlines alone are given here. In the first experiment cold alcohol was followed by hot alcohol, and then by ether. In the second case the more rational method of cold extraction, first with ether and then with alcohol, was carried out: this method, which seems to give the best results, has the advantage of furnishing a good idea of the amount of 'free' and 'masked' phosphatide and other substances; at the same time the necessary manipulation does not tend to cause a disintegration of the extracted substances.

Experiment I

					Sap. value
(1) Cold alcohol extract	= 18·2 grm.	Phosphatide	= 17·0984	mixed	= 262·3
		Fats, &c.	= 1·1216		
(2) Hot alcohol extract	= 11·3692 grm.	Phosphatide	= 8·4186		= 256
		Fats, &c.	= 2·9506		= 141·7
(3) Cold ether extract	= 2·9608 grm.	Phosphatide	= 1·8392	mixed	= 220
		Fats, &c.	= 1·1216		

Total extract	= 32·5300 grm.	Phosphatide	= 27·3562 grm
		Fats, &c.	= 5·1938 grm.
Phosphatide	= 84	per cent. of total extract.	
Fats, &c.	= 16	per cent. of total extract.	

Experiment II

Liver 20 grm. dried substance

Ether extract	Fat, &c.	= 1·6112 grm.	Proportion of Fat to Phosphatide = 2 : 1
	Phosphatide	= 0·8410 grm.	
Alcoholic extract	Fat, &c.	= 0·1176 grm.	Proportion of Fat to Phosphatide = 1 : 20
	Phosphatide	= 2·1914 grm.	

This liver showed fat distinctly on examination with the naked eye, and the total amount of neutral fat was somewhat large. On the other hand, it will be noticed that the alcoholic extract contained about 95 per cent. of phosphatide; the amount of ether-soluble fat other than phosphatide in the liver is probably intimately connected with the digestive processes, and is a much more variable constituent of the liver than the more complex phosphatides. This acetone-soluble 'fat' would likely be reduced in amount as the result of a period of starvation. In short, it would seem that the essential fat of the liver, and probably of certain other organs, is really phosphatide, and under certain circumstances, if care be taken to avoid disintegration during the process of extraction, it may be practically the only one found in any appreciable amount in the combined part of the 'fat.'

THE OSMOTIC PRESSURE OF LIQUID FOODS

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One of the admitted functions of the stomach is the osmotic equilibration that takes place between the blood and the fluid food swallowed. Hypertonic solutions are diluted and hypotonic solutions have salts, etc., added until isotonicity is attained, though it may be a matter of debate whether the diluting (or concentrating) fluid is physiologically secreted or is due to physical diffusion. That the lining cells of the mucous membrane of stomach and gut would be injured by prolonged contact with a hypertonic fluid may be stated *a priori*. Even the mucous membrane of the mouth is open to injury in this way—witness the 'roughness' produced when a piece of confectionery is retained for a few minutes between the teeth and cheek. Also the discomfort which follows the intake of such substances as strong salt solutions, very strong soups, or peptone solutions which 'irritate' the stomach, is thus easily explainable.

The object of the present research was to determine the osmotic pressure of the fluids ordinarily admitted to the stomach, and at the same time to discover whether the sense of taste afforded us any guidance in the choice of fluids with reference to their osmotic pressure, more particularly as regards the rejection of the hypertonic. Of the foodstuffs ordinarily eaten, the vast majority are in solid or gelatinous or colloidal form. To such substances the consideration of osmotic pressure cannot apply. The actual fluid foods admitted to the stomach are milk, the ordinary beverages, fruit juices, beef-tea, meat extracts and soups. In tea, coffee, or cocoa there is usually a sugar addition which varies with personal taste, whilst in beef-tea, soups, etc., common salt is invariably an added ingredient. The osmotic pressure of milk has been determined so frequently that I have not thought it necessary to make any confirmatory experiments. In the case of soups the 'salting to taste' was carried out by a laboratory attendant, who was not aware of the

purpose of the research. The Beckmann freezing point method was employed throughout. A mixture of ice and salt water was used to produce the requisite cold, but care was taken to prevent excessive supercooling. In none of the recorded readings was the degree of supercooling more than about 1·5° C. Crystallisation was started by inoculation with a fragment of frozen distilled water. The stirring was carried out by a simple clock-work mechanism. Centigrade scale employed throughout.

Results.

Beverages

Coffee.—(2 spoonfuls of sugar in ordinary breakfast cupful.) (1) Δ 0·341° C.; (2) Δ 0·343° C.

Tea Infusion.—(2 teaspoonfuls, about 12 c.c., of tea in 200 c.c. boiling water.) Allowed to infuse 5 minutes. (1) Δ 0·052° C.; (2) Δ 0·049° C.; (3) Δ 0·050° C.

Tea.—(100 c.c. Infusion, 50 c.c. water, 25 c.c. milk, 10 grms. of sugar.) Tasted 'just nice.' (1) Δ 0·457° C.; (2) Δ 0·458° C.; (3) Δ 0·456° C.

Tea.—Second infusion, made with 200 c.c. more water added to leaves from infusion and allowed to stand 35 minutes. (1) Δ 0·026° C.; (2) Δ 0·025° C.

Lemon Juice.—(As used in Melbourne Hospital.) Strained juice of lemon (one lemon) 33 c.c. in 250 c.c. distilled water. (1) Δ 0·126° C.; (2) Δ 0·125° C.; (3) Δ 0·122° C.

100 c.c. of diluted juice + 1 teaspoonful (5 grms.) cane sugar added. Tasted 'just right.' (1) Δ 0·487° C.; (2) Δ 0·485° C.

Beer.—(Carlton draught XXX Beer.) (1) Δ 2·407° C.; (2) Δ 2·409° C.

Wine.—(Cheap Australian claret.) Cooled to — 5° C., but ice would not separate out.

Wine.—Kept between 77° C. and 80° C. for 35 minutes, and then boiled briskly for about 7 minutes to get rid of alcohol, at end of which time boiling point rose to about 103° C. 75 c.c. of wine subjected to this treatment yielded 45 c.c. (1) Δ 3·240° C.; (2) Δ 3·238° C.; (3) Δ 3·241° C.

Foodstuffs

Treacle.—Diluted with water to 1 in 7 and this solution gave Δ 1·730° C.

Peptonised Milk.—Benger's peptonised milk as used at the Melbourne Children's Hospital, Carlton, Melbourne, Victoria.

Benger's Milk '*A*.'—(Milk 3, water 1, peptonised 20 minutes. Boiled. Sweetened with cane sugar about 1 oz. to 1 pint milk.) One drop gave pink biuret reaction. (1) Δ 0·652° C.; (2) Δ 0·656° C.

Benger's Milk '*B*.'—(Milk 2, water 1, peptonised 20 minutes. Sweetened.) (1) Δ 0·630° C.; (2) Δ 0·628° C.; (3) Δ 0·626° C.

Peptonised Milk.—(Milk 4, water 1, peptonised 20 minutes.) (1) Δ 0·548° C.; (2) Δ 0·546° C.

Soups.—An ordinary soup which had been served up but rejected as unpalatable on account of salt taste. Δ 1·984° C.

A ***Vegetable Soup*** was made of the following ingredients:—Carrot, 100 grms.; parsnip, 110 grms.; turnip, 55 grms.; spring onion, 47 grms.; celery, 25 grms.; parsley, 12 grms.; water (distilled), 1500 c.c. Brought to boil and kept simmering for 2½ hours. Strained. Salted to different degrees.

Vegetable Soup Plain.—(1) Δ 0·374° C.; (2) Δ 0·373° C.; (3) Δ 0·372° C.

Vegetable Soup Salted.—

A. Soup + salt to 4 per cent. Δ 2·757° C.

B. Soup + salt to 2 per cent. Δ 1·536° C.

C. Soup + salt to $\frac{4}{5}$ per cent. (1) Δ 0·851° C.; (2) Δ 0·856° C.

D. Soup + salt to $\frac{2}{3}$ per cent. Δ 0·780° C.

E. Soup + salt to $\frac{1}{3}$ per cent. (1) Δ 0·586° C.; (2) Δ 0·584° C.; (3) Δ 0·582° C.

The verdict of the taster was:—The unsalted vegetable soup possessed a very flat and unsatisfactory taste. A, B, C, and D distinctly too salty. A and B distinctly unpleasant taste. E was about right.

Beef Tea.—Made with about 6 c.c. meat extract (Fitzroy Brand, Queensland manufacture) in 1000 c.c. distilled water.

Beef Tea Plain.—(1) Δ 0·141° C.; (2) Δ 0·139° C.; (3) Δ 0·140° C.

Beef Tea Salted.—

A. Beef tea + salt to 2·5 per cent. (1) Δ 1·626° C.; (2) Δ 1·626° C.; (3) Δ 1·625° C.

B. Beef tea + salt to 1·25 per cent. (1) Δ 0·882° C.; (2) Δ 0·887° C.; (3) Δ 0·886° C.

C. Beef tea + salt to 0·625 per cent. (1) Δ 0·546° C.; (2) Δ 0·544° C.; (3) Δ 0·543° C.

D. Beef tea + salt to 0·416 per cent. (1) Δ 0·419° C.; (2) Δ 0·416° C.

Sample C was much the tastiest—just salted to taste. A and B having too much salt, and sample D and the original not enough salt.

Beef Tea made with about 6 c.c. meat extract in 1000 c.c. boiling distilled water. Δ 0·160° C.

Beef Tea Salted.—Salt added till the flat and unsatisfying taste of the beef tea was abolished, but still no salty taste perceptible.

Taster A. (1) Δ 0·330° C.; (2) Δ 0·331° C.

Taster B. (1) Δ 0·329° C.; (2) Δ 0·330° C.

Beef Tea Over-salted.—Δ 1·922° C.

Sugar Solutions

Dextrose Solutions.—100 c.c. taken into mouth in sips of 25 c.c., each sip kept in mouth $\frac{1}{2}$ minute, spat out; in $\frac{1}{2}$ minute another sip taken, kept in $\frac{1}{2}$ minute, spat out; and so on for four sips.

10 per cent. Dextrose Solution. Δ 1·156° C.

10 per cent. Dextrose Solution Salivated. (1) Δ 1·068° C.; (2) Δ 1·066° C.

5 per cent. Dextrose Solution. Δ 0·566° C.

5 per cent. Dextrose Solution Salivated. (1) Δ 0·536° C.; (2) Δ 0·532° C.; (3) Δ 0·534° C.

Cane Sugar Solution, 20 grms. in 150 c.c. (13·3 per cent). 100 c.c. treated in similar manner as dextrose solution above, 7 c.c. Saliva were added by this process to the 100 c.c. sugar solution, and there was an after secretion for several minutes.

Cane Sugar Solution (13·3 per cent.) Δ 0·868° C.

Cane Sugar Solution Salivated. (1) Δ 0·788° C.; (2) Δ 0·792° C.

Fruit Juices

Lemon.—Weight 140 grms. Peel and connective structure 90 grms. Yielded 40 c.c. strained juice. (1) Δ 0·937° C.; (2) Δ 0·940° C.; (3) Δ 0·939° C.

Orange.—Orange 135 grms. Yielded 50 c.c. strained juice. (1) Δ 1·100° C.; (2) Δ 1·101° C.; (3) Δ 1·100° C.

Pineapple Juice.—From fresh Queensland pineapple. (1) Δ 1·462° C.; (2) Δ 1·464° C.; (3) Δ 1·460° C.

Cocoanut 'Milk.'—(About 150 c.c. were yielded by the nut.) (1) Δ 0·521° C.; (2) Δ 0·518° C.; (3) Δ 0·518° C.

Saline Aperients

Magnesium Sulphate Solution.—(15 grms. in 100 c.c.) Δ 1·136° C.

Balanced Saline Aperient.—(The stronger one) as recommended by Professor W. A. Osborne in a paper in the *Intercolonial Medical Journal* of Australasia, July 20th, 1909. (1) Δ 0·864° C.; (2) Δ 0·862° C.; (3) Δ 0·861° C·

Saliva produced from Sucking Confectionery.—Barley-sugar stick (about 12 grms.) sucked for 15 minutes led to prodution of 72 c.c. Saliva. (1) Δ 1·008° C.; (2) Δ 1·006° C.

Saliva from about 30 grms. boiled cane sugar. Sweetmeat — 100 c.c. (1) Δ 1·488° C.; (2) Δ 1·464° C.

General Conclusions

It will be seen from the above experiments that of all the fluid foods which are admitted to the stomach, alcoholic beverages and fruit juices alone are hypertonic. Further, it may be safely stated that in no case is a fluid admitted in which hypertonicity is due to the mineral ingredients alone. When, therefore, we find the kidney elaborating a fluid (urine) with sufficient saline ingredients to render it hypertonic, we must regard the high concentration of this fluid as so much external work done and of sufficient moment to be taken into consideration in calorimetric experiments on an animal or on the human subject. These experiments also demonstrate that we must ascribe to the sense of taste a distinct *osmotactic* character. Not only is this sense potent in testing the food qualitatively, but also from the quantitative standpoint of molecular concentration. Even those hypertonic fruit juices which are admitted to the stomach are passed, so to say, under protest, for their taste is recognised as astringent or highly acid, and are apt to be followed by a sense of thirst.

The mechanism is faulty, however, when dealing with alcoholic beverages, a fact which we may ascribe to the artificiality of fermented liquors, and their manufacture and consumption being restricted to man only. The great majority of fluid foods are, however, hypotonic, and thus a margin is left for the addition of hydrochloric acid and other constituents of the gastric juice. With regard to alcoholic beverages, it may be stated that a solution of alcohol in pure water, isotonic with the blood, would only be about 1·5 per cent. As this percentage is almost invariably exceeded in fermented (and, of course, distilled) liquors, and as other substances are present in addition, the high osmotic pressure of the beer and wine tested is not surprising.

The association of the raising of osmotic pressure of beverages with

the induction of thirst is made use of in some departments of commerce by the excessive salting of wines and the over-sugaring of 'summer drinks.' In the case of cane sugar, a solution isosmotic with the blood would be about 11 per cent., whereas the fluid which reaches the stomach as a result of even the slow methods of ingestion of sweetmeats, as exemplified in the process of sucking confectionery, is much higher than this, and accounts for the disagreeable after-results very often experienced after over-indulgence in such delicacies.

In conclusion, I desire to express my sincere thanks to Professor W. A. Osborne for his kind advice and suggestions afforded and interest taken in this work.

THE RELATIONSHIP OF DIASTATIC EFFICIENCY TO AVERAGE GLYCOGEN CONTENT IN THE DIFFERENT TISSUES AND ORGANS

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The first demonstration of an enzyme capable of hydrolysing glycogen is associated with the names of von Wittich (1) and of Claude Bernard (2). The brilliant researches of the latter observer, which ultimately led up to the discovery of glycogen in 1855 (3), formed the basis on which the probability of the presence of such an enzyme rested, but the actual proof of its existence was first furnished by von Wittich; a short time afterwards independent evidence was advanced by Bernard, both observers having found the substance in the liver.

These discoveries, however, were not allowed to pass unchallenged, and to Pavy (4) belongs the credit of having settled beyond dispute the fact that the liver really contains a substance of the nature of an enzyme which is capable of converting glycogen and starch into sugar, and acts quite independently of the vitality of the tissue cells from which it is derived.

The presence of such an enzyme both in the animal and vegetable organism is now universally admitted, but its exact function is still in certain quarters a matter of controversy; the fact that glycogen occurs in the body tissues, and that the diastatic enzyme[1] possesses the power of transforming this substance into dextrose and intermediate products, suggests that the normal function is directly associated with the conversion of glycogen into the less complex substance—sugar.

Pavy (5), however, is unable to accept this view, and suggests that the enzyme is really a product of the dead or dying cell which is generated from an inactive pre-existent zymogen. On the other hand, analogy with other intracellular enzymes points to the probability that this diastatic enzyme actually exerts its influence during life in the same manner as

1. The term 'diastatic enzyme' is used in this paper to indicate the substance or substances by which glycogen is transformed into reducing bodies—maltose, dextrose, etc.; it is generally agreed that at least two distinct enzymes participate in the formation of *dextrose* from starch or glycogen.

its activity is evidenced in vitro—as a hydrolyser of glycogen—the difference being solely one of degree, and its activity being called forth in response to some condition not yet understood. That the conversion of glycogen is not regulated by the condition of the animal is suggested by the experiments of Kisch (6) carried out on adult muscle; his results seem to indicate that regulation of sugar formation from glycogen is apparently not brought about by the needs of the tissues for sugar.

If, then, the function of this ferment is to act on the glycogen present in the organs and change it into sugar in response to some unknown body stimulus, it might be expected that the amount of glycogen normally present in any particular organ would bear some general relationship to the richness of that organ in diastase. It has been known for many years that the chief seat of glycogen in the animal body is the liver; the muscles may also show a very considerable amount, while the other organs contain but traces. Consequently the literature of the subject abounds with data concerning the diastatic activity of the liver and muscles, but comparatively few observations dealing with the other organs are available; it has generally been recognised that many or all the tissues of the body contain traces of a glycogen hydrolysing ferment, but until quite recently no attempt has been made to investigate the relationship of average glycogen content to amylolytic efficiency.

A short time ago, however, an interesting series of observations have been undertaken by Mendel and Saiki (7); for their experiments muscle and liver of pig embryos of various sizes was utilised. In the case of the liver it was found that the diastatic activity increased markedly with the growth of the embryos, while in the case of muscle, which showed in general a well-marked initial activity, the increase was not nearly so pronounced. Hence it would seem that the diastatic activity of embryonic liver and muscle varies directly with the normal glycogen content, for it has been shown by Lockhead and Cramer (8) that the liver of foetal rabbits is at first very poor in glycogen, but that after twenty-five days a considerable amount is present; again the estimations of Mendel and Leavenworth (9) indicate that in the very young pig embryos no glycogen at all may be found. Thus, the foetal liver is at first very poor in glycogen, and apparently also in glycogen hydrolysing substances, while on the other hand the embryonic muscles may contain a fair amount of glycogen even at very early age, but tend to show only slightly increased amounts as age advances; in correlation with this slight rise in glycogen content the increase in diastatic power is correspondingly slight.

So far, therefore, as the subject has been investigated there is, in the case of embryonic tissues, undoubted evidence pointing to a relationship between diastatic efficiency and glycogen content, but whether varying diastatic activity is directly dependent on the relative glycogen content, or whether the increase of diastatic power constitutes a factor which directly influences the amount of glycogen, is still obscure. An answer to this question might help us in attributing its proper function to the diastatic enzyme, for it is not impossible that its chief sphere of action during life is synthetical rather than destructive. If the view entertained by several authorities with regard to glycogen is correct—that under normal circumstances it does not pass on to any marked extent to form dextrose, but is utilised in other ways—then it might perhaps be expected that the measurement of post-mortem amylolytic activity in an organ would afford some clue to its glycogenetic power during life. It will be shown, however, that the amount of diastatic enzyme actually found in different adult tissues bears no apparent relationship to the glycogen content of the same tissues; it is thus obvious that amylolytic efficiency and glycogen storage do not necessarily go together. It might be argued that the comparative absence of glycogen in an organ did not of itself imply the inability of that organ to form glycogen, for the latter substance might be formed and carried away in the blood; or again it might be immediately utilised at the seat of formation by the tissue cells. That the blood does not carry the glycogen as such from an organ is suggested by the very low glycogen content of this fluid, and the conception of a simultaneous process of formation and destruction of glycogen by which this substance might be kept at a minimum, can hardly be entertained if we accept the theory that it is as dextrose that carbohydrate is utilised by the tissues; such a procedure would be quite out of harmony with the ordinary methods of nature, for in that case formation of glycogen would be but a useless step; only on the theory that glycogen is utilised as such by the tissue cells could the latter possibility be entertained.

This apparent lack of correlation between glycogen content and glycogen hydrolysing capacity makes it much more difficult to understand what the amylolytic activity as evidenced post-mortem really means in connection with the vital processes. It may well be that these enzymes exert their destructive action chiefly as the result of some interference which threatens the life of the organ.

It is well known that the kidney, for instance, contains normally

but a very small amount of glycogen, and yet it is often found to be much richer in diastatic enzyme, per unit weight, than any organ in the body. On the other hand, muscle which generally contains a fair amount of glycogen is often very weak in diastatic effect. The following figures, taken from an experiment recorded by Pick (10), are of interest with regard to this point: they indicate the results of some comparative estimations of amylolytic activity of different adult tissues.

It was found that

100 grms. kidney digested 2·37 grms. glycogen in 3 hours.
100 grms. liver digested 0·69 grms. glycogen in 3 hours.

100 grms. blood digested 0·31 grms. glycogen in 3 hours.

Here it is seen that kidney possessed between three and four times the diastatic power of liver and about eight times that of blood. Experiments made by the writer gave in general very similar results, and showed that almost invariably the kidney is the organ which possesses, weight for weight, the greatest amylolytic efficiency. Often other organs were also found to have quite a marked effect. It is interesting to note that in the case of another endoenzyme—erepsin—it has been shown by Vernon that the kidney possesses far and away a greater ereptic action than any other tissue, with the exception of small intestine. The richness of the kidney in these and perhaps other enzymes is not easily explained in the light of our present knowledge of vital processes.

In order to demonstrate the comparative efficiency of the different organs the following procedure was adopted.

Estimation of Amylolytic Activity

The majority of observers conducting research on amylolytic enzymes have chosen as an index of activity the amount of reducing substances formed in a given time. Either the dried tissue or the expressed tissue juice was incubated with a starch or glycogen solution of known strength, and subsequently the extent to which reducing substances had been formed estimated by Fehling's or some similar alkaline copper solution.

This method supplies information as to the activity of the enzyme in terms of its power of forming products which reduce copper solutions, but affords no information as to the relative amounts of these substances actually present—dextrose, maltose, etc. For general comparative work, however, such detailed information is not necessary, and this method is quite suitable.

In other cases the amount of glycogen present before and after incubation was calculated, and the difference taken as an index of amylolytic action. This again merely indicates the power of the enzyme to transform glycogen into substances soluble in alcohol, but gives no indication as to the extent to which this change has been carried on towards the formation of the final products. As exact estimation of glycogen, especially where many experiments have to be undertaken, is a tedious process, a good indication can be obtained by substituting starch for glycogen and testing with iodine in order to ascertain when the starch ceases to give its characteristic iodine reaction. This method has been used and recommended by Wohlgemuth (11), but in my experiments on dried tissues it was found occasionally to give somewhat indefinite results; only a few experiments, however, were carried out, and so far as they went the results were in general similar to those obtained by the reduction method. On the whole the most satisfactory method was found to be the estimation of the reducing power, and dried tissue was mostly employed; in a few experiments moist fresh tissue was also used.

Method

The method utilised is in general the same as that made use of by many observers. The various organs were obtained as soon as possible after death, freed from adherent fat, and quickly cut up into small pieces; these were washed in normal saline solution in order to free them from blood, dried by being pressed gently in a cloth, and finally passed through a mincing machine. The finely divided substance was placed in a large volume of alcohol and left there for 48 hours. The alcohol was then filtered off and the tissue dried in vacuo over H_2SO_4, and ground to a fine powder by a coffee mill. To 50 c.c. of a 1-2% solution of soluble starch or glycogen, 1 gm. of the dried powder was added, and the mixture placed in the thermostat at 37° and left there for 18 to 20 hours. Small flasks of about 100 c.c. capacity were found to be most convenient, and the utmost precautions were adopted in order to ensure the sterility of all vessels used; toluol, alone or with sodium fluoride, was always added to the fluid. The flasks were thoroughly shaken up from time to time, and next day the contents were boiled in order to coagulate the albumin; after filtration a measured volume (10-30 c.c.) was taken and gradually added to excess of boiling Fehling's solution. After boiling for about 5 minutes, the cuprous oxide was

collected on a weighed Gooch crucible containing asbestos; after thorough washing of the precipitate with distilled water, the crucible was strongly heated in order to oxidise the lower oxide, and the precipitate then weighed as cupric oxide. Parallel experiments with boiled controls were also made. In all cases the ordinary Fehling's solution was employed, diluted with an equal volume of water, and in every comparative experiment the same amount of Fehling's solution was used; this precaution was necessary in order to obtain accurate results, and likely depends on the power of caustic alkali to dissolve small amounts of cuprous oxide.

At first some difficulty was occasionally experienced in filtering a too finely divided cuprous oxide precipitate; it was found, however, that this could be overcome in all cases by boiling both the sugar-containing fluid and the alkali before bringing them together, or by adding the sugar solution very slowly to the boiling Fehling's fluid; by adopting this small precaution a well defined red precipitate was easily obtained in every case, and no difficulty arose in filtering.

Some preliminary experiments showed that this method, when carried out carefully as above described, gave practically identical results in parallel experiments with the same tissue. Wohlgemuth, however, raises the objection to results based on reduction methods, that they do not necessarily indicate the diastatic activity, since there may be present glycolytic enzymes which destroy part of the sugar as it is formed. The evidence for the presence to any appreciable extent of active glycolytic enzymes in the tissues is by no means very convincing, as may be seen by consulting the literature of the Conheim controversy relating to the alleged glycolytic action of muscle juice combined with pancreatic extract. In my experiments in which it is sought to establish the different diastatic powers of the various tissues, it is obvious that glycolytic effect is of most importance from a comparative point of view in cases where low results were obtained. Some experiments made on this point showed that mixtures of sugar and tissue, treated exactly as in the diastatic experiments, did not tend to decrease in strength to any appreciable amount; the results obtained indicate that this factor is of little importance in determining the results of diastatic efficiency calculated in terms of reducing substance. In one experiment the following figures were obtained:—

10 c.c. sugar sol. = 0·1126 grm. CuO.

50 c.c. of this sugar solution digested with 1 grm. of following organs for 20 hours; other 50 c.c. + tissue boiled and used as control:

	Digest CuO (10 c.c. used)		Control CuO (10 c.c. used)		Difference between original sugar solution and digest
Liver	0·1130 grm.	...	0·1109 grm.	...	+ 0·0004 grm.
Heart	0·1121 „	...	0·1082 „	...	− 0·0005 „
Lung	0·1046 „	...	0·1090 „	...	0·0080 „
Muscle	0·1102 „	...	0·1113 „	...	− 0·0024 „

While not excluding the possibility of some slight glycolytic effect, the differences obtained are probably within the bounds of experimental error; at any rate, any glycolytic action that may exist is much too small and indefinite to be of any appreciable account in determining diastatic activity, especially from a general comparative point of view.

Relative Diastatic Efficiency of Different Organs in Terms of CuO

Experiments were carried out with tissues obtained from different animals, and it was found that great variations existed not only as regards animals of different species, but in different animals of the same species. The result of an average experiment in each species of animal examined is given below. In almost all cases where sufficient material was available, two sets of parallel experiments were carried out: by this means the possibility of accidental results due to contamination by micro-organisms was excluded. In a few cases in which the organ was too small to yield 1 grm. dried tissue, ½ grm. was taken and the result calculated for 1 grm.

Experiment I.—Cat.

1 grm. dried tissue + 50 c.c. 2 per cent. starch solution + toluol + NaF digested for 18 hours.

Amount of digest used = 20 c.c.	Result in grms. CuO
Kidney	0·3708
Lungs	0·3008
Liver	0·2720
Heart	0·2566
Stomach	0·2210
Bladder	0·1984
Muscle	0·1448

Here it is seen that kidney shows the greatest diastatic action, while lung comes second and liver third; muscle is apparently weaker than any of the tissues examined. Almost identical comparative results were found in another cat examined, except that the lung was less active.

Experiment II.—Rabbit

1 grm. dried tissue + 50 c.c. 2 per cent. starch solution + toluol. Digested 19 hours.

20 c.c. digest used		Result in grms. CuO
Kidney	=	0·2694
Liver	=	0·2028
Stomach	=	0·1572
Lung	=	0·1546
Bladder	=	0·1348
Heart	=	0·1128
Muscle	=	0·0621

In the case of another rabbit, experiments were made with the fresh tissue in order to test the relative effects immediately after death. The animal was killed by bleeding, and the organs removed as quickly as possible and cut up into small pieces. They were then thoroughly minced up and 1 grm. of each quickly weighed and then digested with the above starch solution + toluol. Only kidney, liver and muscle were used for this experiment.

		Time digested	Amount of digest	Result in grms. CuO
Kidney	5 minutes after death	19 hours	10 c.c.	0·1046
Liver	10 ,, ,,	,,	,,	0·0516
Muscle	12 ,, ,,	,,	,,	0·0294

In order to ascertain whether there was any difference if the organ was not used quite fresh, it was tested again three hours after death, exactly as mentioned above.

Result—Kidney	=	0·1016	grms. CuO
Liver	=	0·0584	,,
Muscle	=	0·0318	,,

In another rabbit the following figures were obtained; same starch solution as above + 1 grm. tissue were used, and digestion went on for 20 hours 30 minutes:—

20 c.c. digest used.

Kidney	10 minutes after death	=	0·1872	CuO
	4 hours ,,	=	0·2000	,,
Liver	5 minutes after death	=	0·0640	,,
	4 hours ,,	=	0·0599	,,
Lung	14 minutes after death	=	0·0576	,,
	4 hours ,,	=	0·0521	,,

From these results it appears that tissue does not lose any of its activity on standing, at least for a few hours; other experiments show that no change is apparent after standing for a very much longer time.

Here, again, it is seen that kidney possesses the most marked amylolytic efficiency, while liver has a very much less marked effect and muscle comparatively little. In one rabbit, however, it will be noticed that liver tissue was twice as active as in the next case, while muscle was about equal in both. In the last case, lung is as effective as liver, though lung at most contains only traces of glycogen under normal circumstances. In another case it was found that muscle was somewhat richer in diastatic ferment than liver.

Here, when treated exactly as other dried tissues for 18 hours, it was found that 20 c.c. digest gave the following figures: –

Kidney	=	0·2264 grms. CuO
Liver	=	0·1136 ,,
Muscle	=	0·1200 ,,
Lung	=	0·0716 ,,

These results indicate sufficiently well the marked fluctuations that may exist in the corresponding tissues of the same species of animal.

Experiment III

Pig

In this case only one experiment was made, and the lung gave higher results than any of the other tissues examined. Liver was also somewhat more active than kidney, while skeletal muscle was lowest of all. In this experiment the tissues were used fresh in the moist state, and glycogen taken instead of starch.

2 grms. fresh tissue + 50 c.c. 2 per cent. glycogen + toluol + NaF. Digested 16 hours.

20 c.c. digest used.	Result in grms. CuO
Lung	0·3128
Liver	0·2296
Heart	0·2246
Kidney	0·2190
Muscle	0·1628

In many experiments carried out on animals of other species, it was often found that lung had a relatively high value.

Experiment IV

Dog

The figures obtained from two dogs examined showed that kidney was again most effective, but the difference was not so marked as in some other animals. It is likely that an increase in the number of experiments would have shown greater variations than are here indicated.

1 grm. dried tissue + 50 c.c. 2 per cent. starch solution + toluol. Digested 20 hours.

10 c.c. digest used.	Result in grms. CuO
Kidney	0·1612
Liver	0·1322
Lungs	0·1288
Stomach	0·1051
Bladder	0·1214
Spleen	0·1333
Muscle	0·1051
Small intestine	0·1850[1]

1. In all cases examined small intestine gave a high figure, but as this result is obviously complicated by the effect of the probable presence of ordinary pancreatic juice, this tissue has not been included in the other tables.

Experiment V

Sheep

A very considerable number of experiments were conducted with tissues derived from this animal, and, as in other cases, marked variations were observed, both in the activity of corresponding organs of different animals and in the relative efficiency of the different tissues derived from the same animal. Sometimes kidney was found to be very much more active than liver, or any other organ; in other cases liver and kidney displayed practically an equal effect. In a few cases the heart muscle exerted but very slight diastatic action. Some of the variations obtained are indicated by the following figures from two different animals:—

1 grm. dried tissue + 50 c.c. 1½ per cent. starch solution + toluol + NaF. Digested 20 hours.

20 c.c. digest used.		Result in grms. CuO
	(1)	
Liver	=	0·1876
Kidney	=	0·1796
Muscle	=	0·1414
Heart	=	0·1384
Lungs	=	0·1204
	(2)	
Kidney	=	0·2576
Liver	=	0·1846
Lungs	=	0·1642
Stomach	=	0·1401
Muscle	=	0·0920
Heart		0·0741

A few investigations were carried out with sheep's tissues in order to ascertain whether there was any change in diastatic activity after the fresh moist tissue had stood for a considerable time at room temperature or in a cold chamber.

2 grms. liver were taken at different times after death and digested with starch solution in the usual way, under exactly similar conditions. The results indicate that liver does not lose its full diastatic activity for a very considerable time:

		Result in CuO given by 10 c.c. digest
½ hour after death	=	0·0852
24 hours ,,	=	0·0754
86 ,, ,,	=	0·0835

In another case liver was left for several days in alcohol, part being taken out at different times and dried in the usual way. No difference could be detected between liver treated for 24 hours with alcohol and the same liver treated for four days; the figures given are merely comparative, the same amount of dried tissue being used in each case:—

		Result in grms. CuO
24 hours in alcohol	=	0·0876
48 ,, ,,	=	0·0851
96 ,, ,,	=	0·0836

Samples of powdered tissue kept in a dry state did not seem to have changed in the slightest degree after several months. No indication of any difference in the action of the liver when used as soon as possible after death, and a very considerable time afterwards, was observed, though it is possible that this may not obtain for the glycogen *actually deposited in the liver itself* when acted upon by the cells *immediately* after the death of the animal.

That diastatic enzymes in general are exceedingly stable bodies, especially when protected from moisture, is proved by many researches. Thus Sehrt found active diastatic ferments in the tissue of mummies several thousand years old, and lately it has been shown by White (12) that the seeds of such cereals as wheat, maize and barley contain diastatic enzymes, which, if stored dry, retain their activity for twenty years or more; that these substances are necessarily comparatively stable bodies is obvious from the observation that barley diastase remains unaffected in efficiency after being subjected to temperatures varying from − 200° C. to + 138° C.

These facts indicate that observations on human tissues which can generally be obtained not sooner than 18 to 24 hours after death, probably give quite a fair picture of what would be produced by fresh tissues. In the cases of normal organs examined, the results were generally of the same order as those obtained for other animal tissues. Kidney was generally much more active than any other part, liver being second and lung third. Muscle was again about the bottom of the list; great variations were, however, in evidence, and only a few cases were investigated.

Tissues were also obtained from two patients who had died from diabetes; it has been shown by Bainbridge and Beddard and by others that diastatic activity is present in diabetes, but the relative power of the different organs has not been investigated. In these two cases, however, no appreciable variation from the normal was observed; in No. 1 the marked effect of the kidney over all the other organs is well brought out.

Experiment VI

DIABETIC TISSUES

1 grm. tissue + 50 c.c. 1 per cent. starch + toluol + NaF. Digested 20 hours.

30 c.c. digest used. No. 1

		Result in grms. CuO
Liver	=	0·1191
Kidney	=	0·2642
Muscle	=	0·0959
Heart	=	0·0820
Lungs	=	0·2214

No. 2

Kidney	=	0·1498
Liver	=	0·1089
Lungs	=	0·1186
Muscle	=	0·0707

In these two cases, at any rate, it would seem as if the diabetic condition had at least no tendency to cause diminished diastatic activity; obviously many more cases would be required before any definite statement could be made.

GENERAL SUMMARY

Just as the present investigation was almost finished, several papers by Wohlgemuth (13) on tissue diastases appeared. One contribution dealing with the relative diastatic power of certain organs of the rabbit—liver, kidney, muscle—states that kidney was most powerful, followed by muscle and liver in the order mentioned. The figures given show fairly marked variations, and in some cases liver and muscle appeared to be about equal; sometimes muscle was more powerful than liver.

From these investigations, and those given in this paper, it is quite obvious that there are considerable variations in the relative diastatic activity displayed by different animals; at the same time there is no very definite relationship between the results obtained for the different tissues of two animals of the same species. The experiments given above are intended to indicate chiefly the comparative results obtained with animals of the same species, for sometimes different samples of soluble starch were made use of. At first it was intended to make comparative observations on many different species, but the extent to which variations manifested themselves in animals of the same species indicated that such an investigation would lead to no definite results unless a very great number of animals were used.

The results obtained, however, give sufficient data for an answer to the question of the existence of correlation of diastatic efficiency to glycogen content.

Just as the liver normally contains the most glycogen, and the muscles a considerable quantity, while such organs as the kidney, bladder and lungs contain but traces, it might be expected that these same organs would show diastatic power in the order of their glycogen storing power. This, however, is not the case, and sometimes an organ containing at most but a mere trace of glycogen—e.g. lung shows more marked amylolytic power than liver; again, muscle may contain less ferment than any other organ.

It is obvious from these results that *there exists no definite correlation between glycogen content and diastatic efficiency in the case of adult tissues.*

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THE OSMOTIC PRESSURE OF THE EGG OF THE COMMON FOWL AND ITS CHANGES DURING INCUBATION

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Having shown in a previous paper[1] that the blood and eggs of birds are not isotonic, it seemed of interest to study the changes, if any, taking place during incubation, thus tracing the pressure variations from germ cell to chick. This difference in osmotic pressure between the blood and the egg was quite unexpected: its magnitude may be seen from the following table, in which Δ denotes the depression of freezing point of the fluid below that of pure water and P stands for the osmotic pressure, which was calculated from the formula $P = 12{\cdot}06\ \Delta - 0{\cdot}021\ \Delta^2$.

Bird	Number of experiments	Δ	P in atmospheres
Common fowl (*Gallus bankiva*)	15	0·607° C.	7·31 Blood
	12	0·454° C.	5·47 Egg
Duck (*Anas*)	8	0·574° C.	6·92 Blood
	9	0·452° C.	5·45 Egg
Goose (*Anser*)	4	0·552° C.	6·65 Blood
	1	0·420° C.	5·06 Egg

The above difference in osmotic pressure is accounted for by the diminution in the inorganic salts of the egg as compared with the blood serum. This was ascertained by estimating the chlorides in egg-white and plasma by Volhard's method, after incineration, but as only 0·03 to 0·02 grm. of chlorine was found in the ash owing to the small quantity of material available, the following table is not of great accuracy:

DUCK

Plasma, per cent. chlorine		Egg white, per cent. chlorine
0·278	...	0·080
0·276	...	0·088
0·312	...	0·088
	...	0·104
Mean 0·287 %		Mean 0·090 %
As NaCl 0·473 %		As NaCl 0·148 %

To examine the changes during incubation, eggs were placed in a Hearson egg-incubator and maintained at 40° to 41° C. Samples were taken out at intervals, and their freezing point determined with a Beckmann thermometer, care being taken to avoid freezing out of the

[1] *Proc. Royal Dublin Soc.*, Vol. XII (N.S.), May 1909.

solvent, water, thus leaving a too concentrated solution. The zero of the thermometer was re-determined frequently, both in the apparatus used and in powdered ice and water. Vigorous and continued stirring is necessary in dealing with viscous liquids such as eggs. It is to be regretted that, possibly owing to the lateness of the season—August and September—most of the embryos had died before the shell was broken. This is not a very serious drawback, however, as owing to the variations in the freezing points of fresh eggs of Gallus, from 0·427° C. to 0·480° C. in a dozen determinations, quantitative results proportional to the time of incubation are not to be expected. The experiments are tabulated below:—

Period of incubation Days	Hours	Embryo present Δ	No embryo Δ	Musty eggs Δ
4	17	0·480° C.	—	—
4	17	0·458°C.	—	—
7	22	0·605° C.	0·532° C.	0·620° C.
11	18	0·565° C.	—	0·656° C. embryo present
13	17	0·605° C.	0·538° C.	—
14	17	0·565° C.	—	—
14	17	0·563° C.	—	
14	22	0·590° C.	0·535° C.	—
14	22	—	0·550° C.	—
17	22	0·570° C.	0·538° C.	—
17	22	0·590° C.	—	
18	17	0·611° C.	—	0·633° C. embryo present
18	19½	0·598° C.	0·533° C.	—
19	22	—		0·658° C. embryo present
19	22	—	—	0·680° C. embryo present

Control egg, in room at 15° to 20° C. for twenty days Δ = 0·480° C.

In these determinations the whole of the liquid contents of the egg were mixed, the embryo being rejected, and 12 to 15 c.c. poured into the tube containing the thermometer. It may be seen from the table that there is a rise in the numerical value of the depression of freezing point throughout the period of incubation, the final value, 0·590° C. to 0·611° C., being about that given by the blood, 0·591° to 0·662° C., mean 0·607° C. On the other hand, eggs containing no embryos show a similar rise, but to a much less extent; apparently, as the figures range from 0·532° to 0·550° C., there is an initial rise due possibly to evaporation, although the egg chamber is nearly saturated with water vapour. Thus the rise in osmotic pressure during incubation may be divided into two parts:

(*a*) The rise, as shown by unfertile eggs, probably due to evaporation.

(*b*) The rise due to metabolism of the embryo.

In connection with the latter it is to be noted that both yolk and white become much less viscous during incubation if there is a developing embryo, probably owing to the presence of an enzyme liquefying the reserve materials.

Eggs with or without embryos, but which were musty, gave high values in every case, 0·680° C. being the maximum obtained, though it is quite reasonable to expect that if bacterial action had been more extensive a much higher figure might have been reached. More attention was paid to this effect of bacteria in the second series of experiments than in the first, so this, combined with the impossibility of detecting putrefaction in its initial stages, may account for some of the high values of Δ obtained for eggs with embryos in the early stages of incubation.

The difference in osmotic pressure between the blood and eggs of birds, together with the gradual rise in pressure of the egg to approximate isotonicity with the blood, may, perhaps, be accounted for by the following speculation. There is much evidence that the ontogeny of an organism is a more or less abbreviated repetition of its phylogeny; by extending this view, based on morphological grounds, to physiology, there is reason to believe that birds are descended from ancestors with a lower osmotic pressure, about five or five and a half atmospheres. Fossil remains point to the reptilia as the class from which birds developed. It remains to examine the osmotic pressures of the blood of this group. The following determinations are available:—

	Δ	P	
Thalassochelys Caretta, L.	= — 0·615	...	Bottazzi and Ducceschi [1]
" "	= — 0·602	...	Rodier [2]
Emys europoea (freshwater species of above)	= — 0·463 to — 0·485	...	Bott. and Ducc.

Thus the osmotic pressure of the blood of the only freshwater reptile which I have been able to see recorded, is not very different from that of the eggs of birds, being, in fact, within the limits of variation. It may seem fanciful to regard the osmotic pressure as a hereditary character transmitted with great regularity, but such a possibility seems well worth serious attention in view of the high elaboration of the organs regulating this pressure in all the vertebrates, a regulation not quantitative only, but qualitative. In this connection Loeb's researches (see 'Dynamics of Living Matter') show how marked is the effect of a qualitative difference in the salts present upon developing

[1] Quoted from Rodier.

[2] Station Zoologique d'Arcachon. 1899.

embryos. The whole question of the constancy of osmotic pressure is discussed at length by E. H. Starling (see 'Fluids of the Body'), where Macallum's interesting views on the origin of the blood plasma salts from the waters of a pre-Cambrian ocean are also considered. So apart altogether from the, possibly misleading, agreement in osmotic pressure with certain reptilia, it seems not unreasonable to suppose that birds are descended from a stock which possessed a considerably lower osmotic pressure.

Turning now to the egg membranes which are easily observed, the existence of a fair degree of semi-permeability may be demonstrated by the experiment figured by Bergin and Davis ('Principles of Botany'), in which a chip having been taken out of the rounded end of an egg, over the air space, without piercing the outer membrane, a glass tube of narrow bore is inserted into the pointed end and cemented in position. The contents of the egg will slowly rise in the tube to a height of over a metre when the egg is placed upright in a few centimetres depth of distilled water. If the yolk be pierced it will colour the column, which will stay at its upper limit for several days and then slowly sink. That this membrane is somewhat permeable to sodium chloride may be seen by adding a few drops of silver nitrate solution to the water in which the egg is standing, when a turbidity or precipitate is produced. If, however, the outer membrane be pierced, the rise in the tube will only amount to a few centimetres, for the inner membrane is much more permeable than the outer, as seen by the silver nitrate test. It is to be noted that, while the two membranes are in contact throughout the greater part of the egg, at the blunt end they are separated by the air-space.

Acetic acid also penetrates the two membranes, for if an egg be placed in the dilute acid till the shell is dissolved, and then washed and placed in distilled water, it will be found that the egg swells greatly—to nearly twice its former volume, in fact. The water surrounding it becomes acid, even after numerous changes. If now the egg be placed first in a strong solution of sodium chloride, and then in water, it will swell again, and in this condition may be freely handled without risk of puncture. If the membrane be cut after the shell is dissolved, the egg will be found to be coagulated by the acid, having the appearance of having been boiled.

The yolk is also enclosed in a delicate membrane with some degree of semi-permeability, for on carefully breaking a fresh egg into water, and rinsing to remove the white, the yolk swells considerably and becomes a

paler yellow with a turbid appearance. The germinal disc soon disappears from sight, apparently sinking into the yolk, which by the dilution is now of a lower specific gravity, in which the disc can no longer float. This is evidence that the disc is surrounded by a membrane either impermeable, or more probably less permeable, to water than the yolk membrane. In this distended condition the yolk membrane is very delicate, being ruptured by the weight of its own contents if the water be drawn off from around it. In spite of this slighter permeability of the germinal disc as compared with the yolk membrane, there seems to be no doubt that the disc is normally approximately isotonic with the egg as a whole.

In the above experiment I have been unable to observe whether the disc really sinks into the yolk; it becomes lost to view, but it is just possible that in the imbibed condition it may not be practicable to distinguish it from its surroundings.

That the germinal disc is not necessarily absolutely isotonic with the fluid surrounding it, but is rather in a state of osmotic equilibrium with it, seems extremely likely from the researches of Moore and Roaf (Bio-Chemical Journ., p. 55, Jan. 1908) on the equilibrium between the cell and its environment. These authors showed that there was normally a difference of 0·02° to 0·03° C. between the freezing point of the serum and red blood corpuscles of the pig, and that diluting the blood affected serum and corpuscles unequally. The work of Dakin on the variations in the osmotic pressure of marine vertebrates by change in the external medium (Bio-Chemical Journ., p. 473, Dec. 1908), shows that in these cases also the systems are in equilibrium rather than isotonic.

Summary

The osmotic pressure of the egg of *Gallus bankiva*, as calculated from freezing point depressions, rises during incubation from about 5·5 atmospheres to about 7·3 atmospheres, the latter value being approximately that of the osmotic pressure of the blood of the same bird.

Bacterial action during incubation may cause the pressure to rise to over eight atmospheres. The view is put forward that birds are descended from organisms with an osmotic pressure of five atmospheres or less.

I have much pleasure in thanking Professor A. F. Dixon for his advice and the loan of apparatus, and also Professor H. H. Dixon for permission to carry out the work in the School of Botany.